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GENETIC TOXICITY ASSESSMENT OF CHLOROPENTAFLUOROBENZENE

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TECHNICAL REVIEW AND APPROVAL

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The experiments reported herein were conducted according to the "Guide for the Care and Use of Laboratory Animals," Institute of Laboratory Animal Resources, National Research Council.

This report has been reviewed by the Office of Public Affairs (PA) and is releasable to the National Technical Information Service (NTIS). At NTIS, it will be available to the general public, including foreign nations.

This technical report has been reviewed and is approved for publication.

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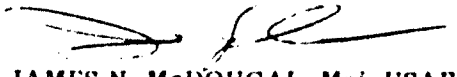

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13. ABSTRACT (Maximum 200 words)

Chloropentafluorobenzene (CPFB), a substituted halogenated benzene, was evaluated in *in vitro* bioassays to assess its potential genotoxic activity. The assays conducted were the Ames Salmonella/microsomal mutagenicity assay, the Chinese hamster ovary (CHO) gene mutation assay, the CHO/ sister chromatid exchange (SCE) and chromosome aberration assay, the BALB/c 3T3 cell transformation assay, and an *in vivo/in vitro* unscheduled DNA synthesis assay. CPFB did not demonstrate genotoxic potential in any assays with the exception of the SCE assay in the presence of metabolizing enzymes. Thus the results of the test battery would predict little or no genetic risk from CPFB.

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PREFACE

The research reported in this document was conducted by Hazleton Laboratories America, Inc. under a subcontract to NSI Technology Services Corporation in support of the Toxic Hazards Research Unit (THRU). The THRU is the contractor-operated effort of the Toxic Hazards Division of the Harry G. Armstrong Aerospace Medical Research Laboratory located at Wright-Patterson Air Force Base, OH. During the initiation and conduct of these studies Melvin E. Andersen, Ph.D.; Lt Col Harvey J. Clewell, III; and Lt Col Michael B. Ballinger served consecutively as the contract technical monitor.

The experimental work reported here was begun on 24 January 1989 and completed 4 September 1989. The genotoxicity assays were conducted at Hazleton Laboratories America facilities in Kensington, MD. The results of their work were reported to NSI in separate reports on each assay. These separate reports were edited by NSI and organized such that the results of each assay are provided in a separate paper. Each paper is authored by the investigator that conducted the study and a final discussion paper is provided to collectively consider the results from the individual studies. The final, unabridged reports received from Hazleton Laboratories with copies of the raw data, Quality Assurance Statements, and Good Laboratory Practice Compliance and Certification Statements for each of the studies are archived in the Quality Assurance Archives of the THRU.

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SECTION 1

INTRODUCTION

Kutzman, R. S.

Chloropentafluorobenzene (CPFB) is a fully substituted halogenated benzene that is being considered as a chemical warfare (CW) agent uptake simulant to assess the effectiveness of chemical defense procedures and equipment.

The selection of CPFB as a candidate CW agent uptake simulant was based on several of its properties that were desirable for a CW agent uptake simulant: good detectability in biological materials; desirable partitioning in biological materials, acceptable physical properties (see below); and relative biological inertness (Jepson et al., 1985).

Physicochemical Properties of CPFB

Chemical Formula	C6C1F5
CAS No.	344-07-0
Molecular Weight	202
Boiling Point (°C)	117
Density (g/mL)	1.66
Vapor Pressure (mmHg, 25°C)	14.1
Purity	99% (capillary gas chromatograph with electron capture detector)
Source	Aldrich Chemical Company, Inc. Milwaukee, WI

It is anticipated that when used in training exercises the breath of trainees will be analyzed, for CPFB, following the exercises to estimate what their dose of a chemical agent would have been. To more fully evaluate the safety of this proposed training agent, *in vitro* and *in vivo/in vitro* tests were conducted to determine its genotoxic potential.

The skin sensitization and acute inhalation toxicity potential of CPFB has been investigated and found to be unremarkable. However, it did produce mild skin and conjunctival irritation in treated rabbits (Kinkead et al., 1987). Repeated inhalation exposures of Fischer 344 rats and B6C3F1 mice to 0.25, 0.80, or 2.50 mg CPFB/L of air for 6 h/day, 5 days/week for 3 weeks resulted in a reduced rate in the growth of the rats but not of the mice (Kinkead, et al., 1989). Following these exposures both species demonstrated increased liver weights. Histologically the tissue exhibited mild hepatocytomegaly with increased granular eosinophilic cytoplasm. Bone marrow samples from the exposed mice were evaluated for evidence of CPFB induced genetic changes; all findings were negative.

Although the genotoxic potential of CPFB has been investigated in several studies (Tu et al., 1986; Steele, 1987), the results have been equivocal. Therefore, to further evaluate the potential risk

of genetic damage as a result of exposure to CPFB and to provide data comparable to that of another possible simulant agent, 1,3-dichlorotetrafluorobenzene (Kutzman et al., 1980), an additional genotoxicity test battery was conducted on CPFB.

This report is arranged such that the results of each assay are provided in a separate paper. Each paper is authored by the investigator who conducted the study. A final discussion paper is provided to collectively consider the results from the individual studies. In addition, the report appendices (Appendix A and B) provide information on the efforts to conduct *in vitro* unscheduled DNA synthesis assays in closed glass culture vessels. It was determined that acceptable culture conditions for rat hepatocytes with such vessel demands would require research beyond that needed to address the biological activity of CPFB. The initial work has been included in this report to provide background for investigators who may pursue the development of the appropriate culture conditions.

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SECTION 2

MUTAGENICITY TEST ON CHLOROPENTAFLUOROBENZENE IN THE SALMONELLA/REVERSE MUTATION ASSAY (AMES TEST) PREINCUBATION METHOD

Lawlor, T.E.[a] and Valentine, D.C.[a]

INTRODUCTION

The mutagenic activity of chloropentafluorobenzene (CPFB) was examined using the *Salmonella*/Reverse Mutation Assay (Ames Test), Preincubation Method. This assay evaluates the test article and/or its metabolites for their ability to induce reverse mutations at the histidine locus in the genome of specific *Salmonella typhimurium* tester strains both in the presence and absence of an exogenous metabolic activation system of mammalian microsomal enzymes derived from Aroclor-induced rat liver.

The *Salmonella*/Mammalian-microsome reverse mutation assay (Ames Test) detects point mutations, both frameshifts and/or base pair substitutions, in bacteria. The strains of *Salmonella typhimurium* used in this assay are histidine auxotrophs by virtue of conditionally lethal mutations in their histidine operon. When these histidine-dependent cells (*his-*) are exposed to the test article and grown under selective conditions (minimal media with a trace amount of histidine) only those cells which revert to histidine independence (*his+*) are able to form colonies. The trace amount of histidine in the media allows all the plated bacteria to undergo a few cell divisions: this growth is essential for mutagenesis to be fully expressed. The *his+* revertants are readily discernable as colonies against the limited background growth of the *his-* cells. By utilizing several different tester strains, both base pair substitution mutations and frameshift mutations can be detected. The Ames Test has been shown to be a sensitive, rapid and accurate indicator of the mutagenic activity of many materials including a wide range of chemical classes.

The tester strains used were the *Salmonella typhimurium* histidine auxotrophs TA98, TA100, TA1535, TA1537 and TA1538. In addition to a mutation in the histidine operon, the tester strains contain two additional mutations which enhance their sensitivity to some mutagenic compounds. The *rfa* wall mutation results in the loss of one of the enzymes responsible for the synthesis of part of the lipopolysaccharide barrier that forms the surface of the bacterial cell wall. The resulting cell wall deficiency increases permeability to certain classes of chemicals such as those containing large ring systems (*i.e.* benzo(a)pyrene) that would otherwise be excluded by a normal intact cell wall. The second mutation, a deletion of the *uvr*B gene, results in a deficient DNA excision repair system which

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greatly enhances the sensitivity of these strains to some mutagens. Since the *uvr*B deletion extends through the *bio* gene, all of the tester strains containing this deletion also require the vitamin biotin for growth.

Strains TA98 and TA100 also contain the R-factor plasmid, pKM101, which further increases the sensitivity of these strains to some mutagens. The mechanism by which this plasmid increases sensitivity to mutagens has been suggested to be by modifying an existing bacterial DNA repair polymerase complex involved with the mismatch-repair process.

Tester strains TA98, TA1537 and TA1538 are reverted from histidine dependence (auxotrophy) to histidine independence (prototropy) by frameshift mutagens. TA1535 is reverted by base substitution mutagens and TA100 is reverted by mutagens which cause both frameshifts and base substitutions.

The doses tested in the mutagenicity assay were selected based on the results of a dose rangefinding study using tester strain TA100 and ten doses of test article ranging from 10,000 to 10.0 µg per plate, one plate per dose, both in the presence and absence of microsomal enzymes.

MATERIALS AND METHODS

Type of Assay: *Salmonella*/Reverse Mutation Assay (Ames Test), Preincubation Method (Ames et al. (1975) and Yahagi et al. (1975).)

Test Article: Chloropentafluorobenzene, Lot No. 01721MV
Physical Description: clear, colorless liquid

CPFB was stored at room temperature. A 10% F-68 Pluronic® sclution (w/v in deionized water, sterilized with a 0.45 µm filter) was used as the vehicle and the test article formed a suspension at 200 mg per mL which was the most concentrated stock dilution of test article prepared. This stock suspension was mixed with a tissuemizer for approximately one minute to enhance the homogeneity of the suspension.

Media and Reagents

Top Agar for Selection of Histidine Revertants: Minimal top agar was prepared with 0.8% agar (w/v) and 0.5% NaCl (w/v). After sterilization by autoclaving, the molten top agar was distributed into sterile bottles and stored at room temperature. Immediately before its use in the mutagenicity assay, the top agar was melted and supplemented with 10 mL per 100 mL agar of a sterile solution which contained 0.5 mM L-histidine and 0.5 mM D-biotin.

Minimal Bottom Agar: Bottom agar was Vogel-Bonner minimal medium E (Vogel and Bonner, 1956).

Nutrient Broth: Nutrient Broth used for growing overnight cultures of the tester strains was Vogel-Bonner salt solution supplemented with 2.5% (w/v) Oxoid Nutrient Broth No. 2 (dry powder).

Nutrient Bottom Agar: Nutrient bottom agar (for tester strain culture density determination) was Vogel-Bonner minimal medium E supplemented with 2.5% (w/v) Oxoid Nutrient Broth No. 2 (dry powder).

Exogenous Metabolic Activation

Liver Microsomal Enzymes - S9 Homogenate: S9 Liver homogenate for use in the mutagenicity assay, prepared as described below, was purchased from Molecular Toxicology, Inc., College Park, MD, 20742, Lot # 0264, 39.8 mg of protein per mL.

Species, Strain, Sex, Inducer: Liver microsomal enzymes were prepared from male Sprague-Dawley rats that had been injected with Aroclor 1254 (200 mg per mL in corn oil) at 500 mg/kg. Five days after i.p. injection with the Aroclor, the rats were sacrificed by decapitation, and their livers were excised.

Homogenate Preparation: The preparation of the microsomal enzyme fraction was carried out with sterile glassware and solutions at 0-4°C. The livers were excised and placed in a beaker containing three volumes of 0.15M KCl (3 mL/g of wet liver) and homogenized. The homogenate was centrifuged at 9000 x g for 10 minutes. Small volumes of the supernatant (referred to by Ames as the S9 fraction) were distributed into freezing ampules which were stored at ≤ -70°C.

S9 Characterization: The S9 homogenate was characterized (using the Ames Assay) for its ability to metabolize selected promutagens to their mutagenic forms, as described by deSerres and Shelby (1979).

S9 Mix: The S9 mix was prepared immediately before its use in the mutagenicity assay. One mL of the microsomal enzyme reaction mixture (S9 mix) contained the following components:

H_2O	0.70 mL
1.00M NaH_2PO_4/Na_2HPO_4, pH 7.4	0.10 mL
0.25M Glucose-6-phosphate	0.02 ml
0.10M NADP	0.04 mL
0.2M $MgCl_2$/0.825M KCl	0.04 mL
S9 Homogenate	0.10 mL
	1.00 mL

When S9 was required, 0.5 mL of the S9 mix was added to the preincubation mixture.

Sham S9 Mix: The Sham S9 mix was prepared immediately before its use in the mutagenicity assay. One mL of the Sham S9 mix contained the following components:

H_2O	0.90 mL
1.00M NaH_2PO_4/Na_2HPO_4, pH 7.4	0.10 mL
	1.00 mL

When S9 was not required, 0.5 mL of the Sham S9 mix was added to the preincubation mixture, in place of the S9 mix.

Test System

The tester strains used were the *Salmonella typhimurium* histidine auxotrophs TA98, TA100, TA1535, TA1537 and TA1538. The description of the tester strains that follows is a summarization of the description provided by Ames et al. (1975).

TESTER STRAIN GENOTYPES

Histidine Mutation			Additional Mutations		
*his*G46	*his*C3076	*his*D3052	LPS	Repair	R Factor
TA1535	TA1537	TA1538	*rfa*	*uvr*B	—
TA100	TA98		*rfa*	*uvr*B	+ R

Source of Tester Strains: The tester strains used were received directly from Dr. Bruce Ames, Department of Biochemistry, University of California, Berkeley.

Frozen Permanent Stocks: Frozen permanent stocks were prepared by growing fresh overnight cultures, adding DMSO (0.09 mL/mL of culture) and freezing small aliquots (approximately 1.5 mL) at ≤ -70°C.

Master Plates: Master plates were prepared by streaking each tester strain from a frozen permanent stock onto minimal agar appropriately supplemented with histidine (260 µM), biotin (3 µM), and for strains containing the R-factor, ampicillin (25 µg/mL). Tester strain master plates were stored at approximately 4°C.

Preparation of Overnight Cultures: Overnight cultures were prepared by transferring a colony from the appropriate master plate to a flask containing culture medium. In order to assure that cultures were harvested in late log phase, the length of incubation was determined by spectrophotometric monitoring. Inoculated flasks were placed in a shaker/incubator which was programed to begin operation (shaking, 125 ± 12.5 rpm; incubation, 37 ± 2°C) so that the overnight cultures were in log phase or late log phase when turbidity monitoring began. Cultures were harvested once a predetermined turbidity was reached as determined by a percent transmittance

(%T) reading on a spectrophotometer. Overgrowth of cultures can result in their loss of sensitivity to some mutagens. Cultures were removed from incubation when the target %T was reached.

Confirmation of Tester Strain Genotypes: Tester strain cultures were checked for the following genetic markers on the day of their use in the mutagenicity assay:

rfa Wall Mutation: The presence of the *rfa* wall mutation was confirmed by demonstration of sensitivity to crystal violet. An aliquot of an overnight culture of each strain was overlaid onto plates containing selective media and an antibiotic sensitivity disk containing 10 µg of crystal violet was added. Sensitivity was demonstrated by inhibition of bacterial growth in a zone immediately surrounding the disk.

pKM101 Plasmid R-factor: The presence of the pKM101 plasmid was confirmed for tester strains TA98 and TA100 by demonstration of resistance to ampicillin. An aliquot of an overnight culture of each strain was overlaid onto plates containing selective media and an antibiotic sensitivity disk containing 10 µg of ampicillin was added. Resistance was demonstrated by bacterial growth in the zone immediately surrounding the disk.

Characteristic Number of Spontaneous Revertants: The mean number of spontaneous revertants per plate in the vehicle controls that are characteristic of the respective strains were demonstrated by plating 100 µL aliquots of the culture along with the appropriate vehicle on selective media.

Experimental Design

Mutagenicity Assay: The tester strains used in this study were TA98, TA100, TA1535, TA1537 and TA1538. The assay was conducted using three plates per dose in the presence and absence of microsomal enzymes. Seven doses of the test article were tested, from 10,000 to 10.0 µg per plate (It should be noted that in this report, the doses have been expressed as µg of test article per plate. This reflects the fact that the exposure of the test system to the test article does not cease at the end of the 20 minute preincubation period. A dose of 10,000 µg per plate indicates that the bacteria are exposed to a concentration of 15,400 µg of test article per mL of preincubation mixture for 20 min prior to being combined with 2 mL of overlay agar and being overlaid onto 25 mL of bottom agar.) in the presence of S9 and from 5,000 to 5.00 µg per plate in the absence of S9. Seven doses of the test article were tested along with the appropriate vehicle and positive controls. The doses tested were selected based on the results of the dose rangefinding study.

Frequency and Route of Administration: The test system was exposed to the test article via the preincubation modification of the Ames Test originally described by Yahagi et al. (1975). This methodology has been shown to detect mutagenicity with certain classes of chemicals, such as

nitrosamines or volatile chemicals, which may not be detected in the standard plate incorporation method. All doses of test article, vehicle controls and positive controls were preincubated and plated in triplicate.

Dose Range-Finding Study: The dose range-finding study was performed using tester strain TA100 both in the presence and absence of microsomal enzymes. Ten doses of test article were tested (one plate per dose). The dose rangefinding study was performed using the same methodology as was used for the mutagenicity assay. Cytotoxicity in this study is detectable as a decrease in the number of revertant colonies per plate and/or a thinning or disappearance of the bacterial background lawn. Routinely, the maximum dose selected to be tested in the mutagenicity assay should demonstrate cytotoxicity if possible.

Rationale: The growth inhibitory effect (cytotoxicity) of the test article on tester strain TA100 is generally representative of that observed on the other tester strains and because of TA100's comparatively high number of spontaneous revertants per plate, gradations of cytotoxicity can be readily discerned from routine experimental variation. Also, the cytotoxicity induced by a test article in the presence of microsomal enzymes may vary greatly from that observed in the absence of microsomal enzymes. Therefore, this would require that different test article dose ranges be tested in the mutagenicity assay based on the presence or absence of the microsomal enzymes.

Positive Controls: All combinations of positive controls and tester strains plated concurrently with the assay are listed below.

POSITIVE CONTROL AND TESTER STRAIN COMBINATIONS

Tester Strain	S9 Mix	Positive Control	Conc. per Plate
TA98	+	2-aminoanthracene	2.5 µg
TA98	-	2-nitrofluorene	1.0 µg
TA100	+	2-aminoanthracene	2.5 µg
TA100	-	sodium azide	2.0 µg
TA1535	+	2-aminoanthracene	2.5 µg
TA1535	-	sodium azide	2.0 µg
TA1537	+	2 aminoanthracene	2.5 µg
TA1537	-	ICR-191	2.0 µg
TA1538	+	2-aminoanthracene	2.5 µg
TA1538	-	2-nitrofluorene	1.0 µg

Source and Grade of Positive Control Articles:

2-aminoanthracene (CAS #613-13-8), Sigma Chemical Co., practical grade; 2-nitrofluorene (CAS #607-57-8), Aldrich Chemical Co., 98%; sodium azide (CAS #26628-22-8), Sigma Chemical Co., practical grade: ICR-191 (CAS #1707-45-0), Polysciences Inc., >95% pure.

Vehicle Controls: Appropriate vehicle controls were plated for all tester strains both in the presence and absence of S9. The vehicle control was plated, using an aliquot of vehicle equal to the aliquot of test article dilution plated, along with an aliquot of the appropriate tester strain, on selective agar.

Sterility Controls: Test Article Sterility Determination: In order to determine the sterility of the test article, the highest test article dose used in the mutagenicity assay was checked for sterility by plating a volume equal to that used in the assay on selective agar.

S9 Mix Sterility Determination: In order to determine the sterility of the S9 mix, 0.5 mL were plated on selective agar.

Plating Procedures

The plating procedures employed are similar to those described by Ames et al (1975) and Yahagi et al (1975). These procedures were employed for both the Dose Rangefinding Study and the Mutagenicity Assay.

Test System Identification: Each plate was labeled with a code system which identified the test article, test phase, dose and activation condition.

Test Article Plating Procedure: The test article was diluted and the S9 mix was prepared immediately before their use in any experimental procedure.

When S9 mix was required, 0.5 mL of S9 mix was added to 13 x 100 mm glass, screw-cap, culture tubes, pre-heated to 37 ± 2°C. To these tubes were added 100 µL of appropriate tester strain and 50 µL of vehicle or test article dilution. When S9 mix was not required, 0.5 mL of Sham S9 (0.1M phosphate buffer) was substituted for the S9 mix. Once all components had been added to a tube, it was tightly capped, and after vortexing, the mixture was allowed to incubate for 20 ± 2 minutes at 37 ± 2°C. The caps were then removed and 2.0 mL of molten selective top agar was added to each tube and, after vortexing, the mixture was overlaid onto the surface of 25 mL of minimal bottom agar contained in a 15 x 100 mm petri dish. After the overlay had solidified, the plates were inverted and incubated for approximately 48 h at 37 ± 2°C.

Scoring Plates

Plates which were not scored immediately after the 48 ± 4.8 h incubation period were held at 4 ± 2°C until such time that scoring could occur.

Evaluation of the Bacterial Background Lawn: The condition of the background bacterial lawn was evaluated for evidence of cytotoxicity due to the test article by using a dissecting microscope (Appendix-A). The cytotoxicity was scored relative to the vehicle control plate. In addition to the cytotoxicity, any test article precipitate observed on the plates was also noted at the appropriate dose on the data tables.

Colony Counting: Revertant colonies for a given tester strain and activation condition were counted either entirely by automated colony counter or entirely by hand. If the plates contained sufficient test article precipitate to interfere with automated colony counting, then they were counted manually.

Analysis of the Data: For all replicate platings, the mean number of revertants per plate was calculated and the standard deviation around the mean was also calculated.

Criteria for Determination of a Valid Test

The following criteria must be met for the assay to be considered valid:

Tester Strain Integrity

rfa Wall Mutation: In order to demonstrate the presence of the deep rough mutation, all tester strain cultures must exhibit sensitivity to crystal violet

pKM101 Plasmid R-Factor: In order to demonstrate the presence of the pKM101 Plasmid R-factor, all tester strains must exhibit resistance to ampicillin.

Characteristic Number of Spontaneous Revertants: All tester strain cultures must exhibit a characteristic number of spontaneous revertants per plate in the vehicle controls. The acceptable ranges are as follows:

TA98	10 - 60
TA100	80 - 240
TA1535	5 - 45
TA1537	3 - 21
TA1538	5 - 35

Tester Strain Culture Density: In order to ensure that appropriate numbers of bacteria are plated, tester strain culture density must be greater than or equal to 5.0×10^8 bacteria per mL.

Positive Control Values: All positive controls must exhibit at least a three-fold increase in the number of revertants per plate over the mean value for the vehicle control for the respective strain.

Cytotoxicity

Acceptable Number of Non-toxic Doses: A minimum of three non-toxic doses are required to evaluate assay data.

Evaluation of Test Results

Tester Strains TA98 and TA100

For a test article to be considered positive, it must cause at least a 2-fold increase in the mean revertants per plate of at least one tester strain over the mean vehicle control value for that tester strain. This increase in the mean number of revertants per plate must be accompanied by a dose response to increasing concentrations of the test article.

Tester Strains TA1535, TA1537 and TA1538

For a test article to be considered positive, it must cause at least a three-fold increase in the mean revertants per plate of at least one tester strain over the mean vehicle control value for that tester strain. This increase in the mean number of revertants per plate must be accompanied by a dose response to increasing concentrations of the test article.

RESULTS AND DISCUSSION

Dose Rangefinding Study

Doses of CPFB to be tested in the mutagenicity assay were selected based on the results of the dose rangefinding study conducted using tester strain TA100 in both the presence and absence of S9 (one plate per dose). Ten doses of test article, from 10,000 to 10.0 µg were tested and the results are presented in Table 2-1. Cytotoxicity was observed at 667 µg per plate and above in the presence of S9 and at 333 µg per plate in the absence of S9 as evidenced by the reduced number of revertants per plate and/or the thinning or disappearance of the bacterial background lawn.

Mutation Assay

The results of the dose rangefinding study were used to select seven doses to be tested in the mutagenicity assay. The doses selected for the mutation assay ranged from 10,000 to 10.0 µg per plate in the presence of S9 and from 5,000 to 5.00 µg per plate in the absence of S9. All data generated on the mutagenicity assays were acceptable and all criteria for a valid study were met.

There were no positive increases in the mean number of revertants per plate observed with any of the tester strains either in the presence or absence of S9 (Appendix 2-B).

Under the conditions of this study, the results of the *Salmonella*/Reverse Mutation Assay (Ames Test), Preincubation Method, indicate that CPFB did not cause positive increases in the numbers of histidine revertants per plate of any of the tester strains either in the presence or absence of microsomal enzymes prepared from Aroclor-induced rat liver.

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TABLE 2-1. DOSE RANGEFINDING STUDY FOR CPFB

µg/PLATE	TA100 REVERTANTS PER PLATE			
	WITH S9		WITHOUT S9	
	NUMBER OF COLONIES/PLATE	APPEARANCE OF BACKGROUND LAWN*	NUMBER OF COLONIES/PLATE	APPEARANCE OF BACKGROUND LAWN*
0.00 (Vehicle) (50.0 µL)	131	1	93	1
CPFB				
10.0	103	1	120	1
33.3	86	1	128	1
66.7	109	1	113	1
100	109	1	107	1
333	111	1	97	2
667	97	2	78	2
1000	74	2	94	2
3330	58	3	79	3
6670	81	3	35	4
10000	65	3	0	4

*Background Lawn Evaluation Codes:
1 = normal
2 = slightly reduced
3 = moderately reduced
sp = slight precipitate
mp = moderate precipitate (requires hand count)
4 = extremely reduced
5 = absent
6 = obscured by precipitate
hp = heavy precipitate (requires hand count)

APPENDIX 2-A

BACTERIAL BACKGROUND LAWN EVALUATION CODE

The condition of the background bacterial lawn is evaluated both macroscopically and microscopically (using a dissecting microscope) for indications of cytotoxicity and test article precipitate as follows:

CODE	DEFINITION	CHARACTERISTICS
1	Normal	A healthy microcolony lawn.
2	Slightly Reduced	A noticeable thinning of the microcolony lawn and an increase in the size of the microcolonies compared to the vehicle control plate.
3	Moderately Reduced	A marked thinning of the microcolony lawn and an ease in the size of the microcolonies compared to the vehicle control plate.
4	Extremely Reduced	An extreme thinning of the microcolony lawn and an increase in the size of the microcolonies compared to the vehicle control plate.
5	Absent	A complete lack of any microcolony lawn.
6	Obscured by Precipitate	The background bacterial lawn cannot be accurately evaluated due to microscopic and/or macroscopic test article precipitate.

Evidence of macroscopic test article precipitate on the plates is recorded by addition of the following precipitate code to the code number used to evaluate the condition of the background bacterial lawn.

SP	Slight Precipitate	Noticeable macroscopic precipitate on the plate, however, the precipitate does not influence automated counting of the plate.
MP	Moderate Precipitate	The amount of macroscopic precipitate on the plate would interfere with automated counting, thus, requiring the plate to be hand counted.
HP	Heavy Precipitate	The large amount of macroscopic precipitate on the plate makes the required hand counting difficult.

Example: 4-MP would indicate a plate observed to have an extremely reduced background lawn which had to be counted manually due to the marked amount of macroscopic test article precipitate.

APPENDIX 2-B

SALMONELLA MUTAGENICITY ASSAY RESULTS FOR CPFB

INDIVIDUAL PLATE COUNTS

TEST ARTICLE ID: CPFB
VEHICLE: 10% F-68 Pluronic® (w/v in H_2O)
PLATING ALIQUOT: 50.0 µL

	REVERTANTS PER PLATE															
DOSE/PLATE	TA1535			TA1537			TA1538			TA98			TA100			BACKGROUND LAWN*
	1	2	3	1	2	3	1	2	3	1	2	3	1	2	3	
MICROSOMES: None																
VEHICLE CONTROL	8	8	13	5	3	4	7	5	5	19	9	11	81	78	87	1
TEST ARTICLE 5.00µg	10	6	15	2	5	3	4	2	6	13	17	16	107	77	78	1
10.0 µg	11	11	7	4	2	2	1	8	0	8	14	8	97	115	90	1
50.0 µg	6	9	14	3	9	2	6	5	1	19	17	13	81	88	78	1
100 µg	6	12	7	5	5	5	8	8	7	22	22	19	78	103	73	1
500 µg	10	9	9	3	2	2	4	3	9	10	9	7	62	47	75	2
1000 µg	3	4	9	2	3	1	2	4	0	6	7	7	67	66	46	3
5000 µg	8	3	8	4	4	4	4	4	4	7	9	4	40	46	50	3
POSITIVE CONTROL **	496	502	497	1064	931	999	303	300	310	233	203	209	452	512	500	1
MICROSOMES: Rat Liver																
VEHICLE CONTROL	13	16	16	10	7	3	10	9	12	20	23	24	106	105	115	1
TEST ARTICLE 10.0 µg	9	15	15	6	5	7	20	17	8	22	24	34	107	115	105	1
50.0 µg	13	7	11	5	7	10	16	15	13	22	35	27	129	130	122	1
100 µg	11	10	17	4	5	5	13	14	16	20	23	23	103	110	118	1
500 µg	10	10	8	16	6	8	15	15	20	20	23	22	111	113	89	1
1000 µg	13	7	10	7	7	12	11	18	14	12	19	21	91	100	84	2
5000 µg	13	8	9	2	2	4	7	6	5	17	20	14	90	87	73	3
10000 µg	6	9	12	7	4	4	9	8	15	2	13	11	75	92	100	3
POSITIVE CONTROL ***	129	159	116	85	105	95	726	924	759	861	750	740	888	845	698	1

** TA1535	sodium azide	@ 10 µg/plate	*** TA1535	2-aminoanthracene @ 2.5 µg/plate
TA1537	quinacrine mustard	@ 5 µg/plate	TA1537	2-aminoanthracene @ 2.5 µg/plate
TA1538	2-nitrofluorene	@ 10 µg/plate	TA1538	2-aminoanthracene @ 2.5 µg/plate
TA98	2-nitrofluorene	@ 10 µg/plate	TA98	2-aminoanthracene @ 2.5 µg/plate
TA100	sodium azide	@ 10 µg/plate	TA100	2-aminoanthracene @ 2.5 µg/plate

* Background Lawn Evaluation Codes:

1 = normal; 2 = slightly reduced; 3 = moderately reduced
4 = extremely reduced; 5 = absent; 6 = obscured by precipitate
sp = slight precipitate; mp = moderate precipitate (requires hand count); hp = heavy precipitate (requires hand count)

APPENDIX 2-B (cont)

SALMONELLA MUTAGENICITY ASSAY RESULTS FOR CPFB

SUMMARY OF TEST RESULTS

TEST ARTICLE ID: CPFB
VEHICLE: 10% F-68 Pluronic® (w/v in H_2O)
PLATING ALIQUOT: 50.0 µL

	MEAN REVERTANTS PER PLATE WITH STANDARD DEVIATIONS										
DOSE/PLATE	TA1535		TA1537		TA1538		TA98		TA100		BACKGROUND LAWN*
	MEAN	S.D.	MEAN	S.D.	MEAN	S.D	MEAN	S.D.	MEAN	S.D.	
MICROSOMES: None											
VEHICLE CONTROL	10	3	4	1	6	1	13	5	82	5	1
TEST ARTICLE 5.00µg	10	5	3	2	4	2	15	2	87	17	1
10.0 µg	10	2	3	1	3	4	10	3	101	13	1
50.0 µg	10	4	5	4	4	3	16	3	82	5	1
100 µg	8	3	5	0	8	1	21	2	85	16	1
500 µg	9	1	2	1	5	3	9	2	61	14	2
1000 µg	5	3	2	1	2	2	7	1	60	12	3
5000 µg	6	3	4	0	4	0	7	3	45	5	3
POSITIVE CONTROL **	498	3	998	67	304	5	215	16	488	32	1
MICROSOMES: Rat Liver											
VEHICLE CONTROL	15	2	7	4	10	2	22	2	109	6	1
TEST ARTICLE 10.0 µg	13	3	6	1	15	6	27	6	109	5	1
50.0 µg	10	3	7	3	15	2	28	7	127	4	1
100 µg	13	4	5	1	14	2	22	2	110	8	1
500 µg	9	1	10	5	17	3	22	2	104	13	1
1000 µg	10	3	9	3	14	4	17	5	92	8	2
5000 µg	10	3	3	1	6	1	17	3	83	9	3
10000 µg	9	3	5	2	11	4	9	6	89	13	3
POSITIVE CONTROL ***	135	22	92	12	803	106	784	67	810	100	1

**	TA1535	sodium azide @ 10 µg/plate	***	TA1535	2-aminoanthracene @ 2.5 µg/plate
	TA1537	quinacrine mustard @ 5 µg/plate		TA1537	2-aminoanthracene @ 2.5 µg/plate
	TA1538	2-nitrofluorene @ 10 µg/plate		TA1538	2-aminoanthracene @ 2.5 µg/plate
	TA98	2-nitrofluorene @ 10 µg/plate		TA98	2-aminoanthracene @ 2.5 µg/plate
	TA100	sodium azide @ 10 µg/plate		TA100	2-aminoanthracene @ 2.5 µg/plate

* Background Lawn Evaluation Codes:

1 = normal; 2 = slightly reduced; 3 = moderately reduced
4 = extremely reduced; 5 = absent; 6 = obscured by precipitate
sp = slight precipitate; mp = moderate precipitate (requires hand count); hp = heavy precipitate (requires hand count)

SECTION 3

MUTAGENICITY TEST ON CHLOROPENTAFLUOROBENZENE IN THE CHO/HGPRT FORWARD MUTATION ASSAY

Young, R.R.*

ABSTRACT

The objective of this *in vitro* assay was to evaluate the ability of chloropentafluorobenzene (CPFB) to induce forward mutations at the hypoxanthine-guanine phosphoribosyl transferase (HGPRT) locus in Chinese hamster ovary cells under conditions with and without metabolic activation.

The test material was emulsified in the biologically compatible surfactant Pluronic® F-68 to permit preparation of homogeneous treatment media. CPFB etched polystyrene tissue culture flasks normally used for cell cultivation and treatment. For this reason, cell treatment for the cytotoxicity and mutation assays was performed in sterile glass tissue culture bottles. The cells were exposed to the test material for four hours in the presence and absence of rat liver S9 metabolic activation. The test material remained in an emulsion during the four hour treatment period. Dose-related toxicity was observed both without metabolic activation and with metabolic activation. Mutant frequencies of cultures treated with test material varied randomly with dose within ranges comparable to the mutant frequencies of the concurrent vehicle controls in the nonactivation trial. With S9 metabolic activation, mutant frequencies of treated cultures varied randomly with dose at background levels with the exception of the highest acceptable dose. That culture had a mutant frequency that was significantly elevated over background mutant frequencies of the concurrent vehicle controls. A second activation mutation assay was performed that used a modified dose range to investigate the response observed in the first activation trial. In the second activation mutation assay, mutant frequencies varied randomly with dose and toxicity at levels within the acceptable background range which is 0 to 15 x 10^{-6}. The single significantly elevated mutant frequency seen in the first activation mutation assay appeared to represent normal assay variation. The test material was therefore evaluated as negative for inducing forward mutations at the HGPRT locus in Chinese hamster ovary cells under conditions with and without metabolic activation.

INTRODUCTION

Hypoxanthine-guanine phosphoribosyl transferase (HGPRT) is a cellular enzyme that allows cells to salvage hypoxanthine and guanine for use in DNA synthesis. The HGPRT enzyme utilizes the substrates 5-phosphoribosyl-1-pyrophosphate and hypoxanthine or guanine to catalyze the

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formation of inosine- or guanosine mono-phosphate. If a purine analog such as 6-thioguanine (TG) is included in the growth medium, the analog will be phosphorylated via the HGPRT pathway and incorporated into nucleic acids, eventually resulting in cellular death. The HGPRT locus is located on the X chromosome. Since only one of the two X chromosomes is functional in the female CHO cells, a single-step forward mutation from HGPRT + to HGPRT- in the functional X chromosome will render the cell unable to utilize hypoxanthine, guanine, or TG supplied in the culture medium. Such mutants are as viable as wild-type cells in normal medium because DNA synthesis may still proceed by *de novo* synthetic pathways that do not involve hypoxanthine or guanine as intermediates. The basis for the selection of HGPRT- mutants is the loss of their ability to utilize toxic purine analogs (*e.g.*, TG), which enables only the HGPRT- mutants to grow in the presence of TG. Cells which grow to form colonies in the presence of TG are assumed to have mutated, either spontaneously or by the action of the test article, to the HGPRT- genotype.

The objective of this *in vitro* study was to evaluate the ability of CPFB to induce forward mutations at the HGPRT locus in the CHO-K1-BH_4 Chinese hamster ovary (CHO) cell line as assessed by colony growth in the presence of 6-thioguanine (TG). Testing was performed both in the presence and absence of S9 metabolic activation.

MATERIALS AND METHODS

Test Article:

Chloropentafluorobenzene, 99 + %

Lot Number: 01721MV

Physical Description: Clear, colorless liquid

Indicator Cells

The indicator cells used for this study were Chinese hamster ovary (CHO) cells. The hypodiploid CHO-K1 cell line was originally derived from the ovary of a female Chinese hamster (*Cricetulus griseus*) (Kao and Puck, 1968). Characteristics of the cell line were high clonability (approximately 85%) and rapid doubling time (11-14 h). The particular clone used in this assay was CHO-K1-BH_4. The BH_4 subclone of CHO-K1 cells, isolated by Dr. A.W. Hsie (Oak Ridge National Laboratory, Oak Ridge, Tennessee), has been demonstrated to be sensitive to many chemical mutagens.

The CHO-K1-BH_4 cells used in this study were obtained in October 1982 from Dr. Hsie. Master stocks of the cells were maintained frozen in liquid nitrogen. Laboratory cultures were maintained as monolayers at 37C° ± 1°C in a humidified atmosphere containing 5% ± 1.5% CO_2. Laboratory cultures were periodically checked for karyotype stability and for the absence of mycoplasma contamination. To reduce the negative control frequency (spontaneous frequency) of HGPRT- mutants to as low a level as possible, the cell cultures were exposed to conditions which selected

against the HGPRT- phenotype. Cells were maintained in cleansing medium for two to three days, placed in recovery medium for one day and then returned to culture medium. Cleansed cultures were used to initiate mutation assays from three to seven days after having been removed from cleansing medium.

Media

The cells used during experimental studies were maintained in Ham's Nutrient Mixture F12 supplemented with L-glutamine, antibiotics, and fetal bovine serum (8% by volume), hereafter referred to as culture medium. Cleansing medium used for reducing the spontaneous frequency of HGPRT- mutants prior to experimental studies consisted of culture medium (5% serum) supplemented with 5.0×10^{-6} M thymidine, 1.0×10^{-5} M hypoxanthine, 1.0×10^{-4} M glycine, and 3.2×10^{-6} M of either aminopterin or methotrexate. Recovery medium was similar to cleansing medium except that the aminopterin or methotrexate component was removed and the fetal bovine serum was increased to 8% by volume. Selection medium for mutants was hypoxanthine-free F12 medium containing 4 µg/mL (24 mM) of TG and the fetal bovine serum component reduced to 5% by volume.

Control Articles

Negative (media) controls were performed for each portion of the assay by carrying cells unexposed to the test article through all of the assay operations. In the activation portion of the assay, the negative control cultures were exposed to the S9 metabolic activation mix. A single negative control culture was used in the cytotoxicity assays. A single negative control was added to the first activation mutation assay but not in the other mutation assays.

The test material was a liquid with poor solubility directly in water or culture medium. In order to effectively prepare primary 10X stocks, the test material was emulsified in sterile deionized water that contained ten percent (w/v) Pluronic® F-68 (BASF-Wyandotte, Lot numbers WPHB 500B and WPDJ 546B). The primary test material stocks were then diluted 1:10 into culture medium resulting in varying test material concentrations emulsified in 1% Pluronic® F-68 in the treatment medium. Therefore, concurrent vehicle controls were performed for each portion of the study by exposing cells to 1% Pluronic® F-68 in culture medium. In the activation portions of the study, the vehicle controls were also exposed to the rat liver S9 metabolic activation mix. A single culture was used in the cytotoxicity assays and duplicate cultures were used in the mutation assays.

5-Bromo-2'-deoxyuridine (BrdU) is a chemical that is reproducibly and highly mutagenic to CHO-K1 cells without S9 metabolic activation. BrdU (Sigma Chemical Co., Lot number 81F-0082) was used at a concentration of 50 µg/mL as a concurrent positive control article for nonactivation mutation studies.

3-Methylcholanthrene (MCA) requires metabolic activation by microsomal enzymes to become mutagenic to CHO-K1-BH_4 cells. MCA (Sigma Chemical Co., Lot number 70F-0306) was used at 5 µg/mL as a concurrent positive control article for mutation assays performed with S9 activation.

S9 Metabolic Activation System

The *in vitro* metabolic activation system was comprised of rat liver enzymes (S9 fraction) and an energy producing system, CORE (nicotinamide adenine dinucleotide phosphate, glucose-6-phosphate and an ion mix) prepared in a phosphate buffer. The enzymes were contained in a 9000 x g supernatant from liver homogenate prepared from Sprague Dawley rats treated with 500 mg/kg of Aroclor 1254 five days prior to sacrifice (Molecular Toxicology, Inc., Lot number 0249). The treatment with Aroclor 1254 was used to induce mixed function oxidase enzymes capable of transforming chemicals to more active forms. The S9 and reaction mixture (CORE) were retained frozen at about -80°C until used. The S9 fraction and CORE were thawed immediately before use and combined to form the activation system described below:

Component	Final Concentration in Cultures
NADP (sodium salt)	1.0 mM
Glucose-6-phosphate	5.0 mM
Calcium chloride	2.0 mM
Potassium chloride	6.6 mM
Magnesium chloride	2.0 mM
Phosphate	2.0 mM
S9 homogenate	20.0 µL/mL

The amount of S9 homogenate per culture depends upon the lot of S9 in use at any time. Before use in the assay, each lot of S9 homogenate was tested following receipt. Because the enzymatic activity of S9 homogenate varies among lots, S9 at various concentrations was tested against reference chemicals such as benzo(a)pyrene or 3-methylcholanthrene. The optimum S9 concentration was selected based on induction of HGPRT- mutants in CHO cells, and this amount of S9 was used in all subsequent assays with that particular lot of S9.

Dosing Procedure

In order to achieve as uniform an exposure as possible for cell monolayers treated with the test material in culture medium, Pluronic® F-68 was investigated as an emulsifying agent for the test material. Pluronic® F-68 is a polyalcohol that is frequently used in cell cultures due to its low toxicity and ability to lower surface tension. When a preparation of 50 mg/mL of CPFB in 10% w/v Pluronic® F-68 in deionized water was vigorously agitated for about one minute with a Tissumizer®, a stable, white emulsion was formed. The CPFB remained dispersed into tiny droplets after diluting 1:10 into

culture medium and did not coalesce upon sitting, even though dense droplets settled and collected on the bottom of the container. A brief mixing with a vortex caused complete dispersion.

It was also noted that CPFB scored and softened polystyrene tissue culture flasks, whether placed directly on the surface of the flask or as an emulsion in Pluronic® F-68. Thus, emulsified CPFB appeared to be available to interact with the cells, and only glass containers, pipets and culture bottles were used for this study

A fresh emulsion of CPFB was prepared in 10% w/v Pluronic® F-68 for each dose rangefinding study and mutation assay. A Tissumizer® was used at a setting of 40-50 for 45 sec to 1 min to prepare the emulsions. The initial stocks of CPFB were prepared at between 6.0 mg/mL and 50 mg/mL, and lower concentrations were prepared by diluting the emulsions into 10% w/v Pluronic® F-68. The emulsions were mixed well by vortex before preparing the treatment media. The treatment media were prepared as 1:10 dilutions of the emulsions into F12 culture medium so that the Pluronic® F-68 content was diluted to 1% w/v.

Rangefinding Cytotoxicity Testing

After the selection of 10% w/v Pluronic F-68® as a suitable vehicle, a wide range of test article concentrations was tested for cytotoxicity both with and without S9 metabolic activation. Ten concentrations that spanned a 3-log concentration range were used. The applied doses ranged from 0.005 mg/mL to 5.0 mg/mL. In addition, one negative (media) control and one vehicle control containing 1% w/v Pluronic® F-68 were used in each cytotoxicity assay.

Glass culture bottles with approximately 60 cm^2 of surface area were seeded at 200 cells per flask, allowed to attach overnight (16 to 18 h) and exposed to the test or control article for four hours. A single culture was used for each test or control treatment condition. The cells were then washed twice with Dulbecco's phosphate buffered saline (PBS) and incubated in F12 culture medium for six additional days to allow colony development. Colonies were then fixed in alcohol, stained with Giemsa and counted by eye, excluding those with approximately 50 cells or less. Cytotoxicity was expressed as a percentage of colony counts in treated cultures versus control culture. The preliminary cytotoxicity information was used to select doses for the mutation assays that covered a range of toxicity from nontoxic to highly toxic.

Mutagenicity Nonactivation Assay

The assay procedure was based on that reported by Hsie, et al. (1975), and reviewed by Hsie, et al. (1981), with modifications suggested by Myhr and DiPaolo (1978). The cleansed cells were plated at about 2.4 x 10^6 cells per 60 cm^2 glass tissue culture bottle on the day before dosing. The time between plating and treatment was about 18 hours. Cell cultures were treated with test or control

material for four hours. Cell cultures normally contain at least 4 x 10^6 cells by the time of treatment termination. After treatment, the cell monolayers were washed twice with phosphate buffered saline, trypsinized, and suspended in culture medium. The cell suspension from each dose was counted using a Coulter Counter and replated at 1.5 x 10^6 cells into each of two 150-mm dishes and at 200 cells into each of three 60-mm dishes. The small dishes were incubated for seven days to permit colony development and the determination of the cytotoxicity associated with each treatment. The large dishes were incubated for seven days to permit growth and expression of induced mutations. The mass cultures were subcultured every two or three days during the expression period to maintain logarithmic cell growth. At each subculture the cells from the two 150-mm dishes from each dose were combined and reseeded at about 1.5 x 10^6 cells into each of two 150-mm dishes.

At the end of the expression period (seven days), each culture was reseeded at 2 x 10^5 cells per 100-mm dish (12 dishes total) in mutant selection medium. Also, three 60-mm dishes were seeded at 200 cells each in culture medium to determine the cloning efficiency of each culture. After incubation for seven to ten days, at 37°C ± 1°C in a humidified atmosphere with about 5% CO_2, the colonies were fixed with alcohol, stained with Giemsa and counted to determine the number of TG-resistant colonies in mutant selection dishes and the number of colonies in the cloning efficiency dishes. The colonies were counted by eye, excluding those with approximately 50 cells or less.

Mutagenicity Activation Assay

The activation assay was performed independently with its own set of vehicle and positive controls. The procedure was identical to the nonactivation assay except for the addition of the S9 fraction of rat liver homogenate and necessary cofactors during the four-hour treatment period. The fetal bovine serum content of the medium used for dosing was reduced to 5% by volume. The cofactors consisted of nicotinamide adenine dinucleotide phosphate (NADP, sodium salt), glucose-6-phosphate, calcium chloride, potassium chloride, and magnesium chloride, all of which were in a pH 7.8 sodium phosphate buffer

Data Presentation

The collected data were used to calculate several assay parameters. The chosen combination of raw data and calculated data allows a complete description of events for each treatment condition. The significance of each calculated parameter and its method of calculation are listed below:

Relative Survival to Treatment: This parameter gives the clonal cytotoxicity of each treatment by showing what percentage of the cells were able to form colonies after the treatment period in both the rangefinding cytotoxicity assays and the mutation assays relative to the concurrent vehicle

controls. The average number of colonies in three dishes (seeded at 200 cells each) was determined for each treatment condition.

$$\text{Relative Survival (\%)} = \frac{\text{Average no. of colonies per treated culture}}{\text{Average no. of colonies per vehicle control dish}} \times 100\%$$

Relative Population Growth: This parameter shows the cumulative growth of the treated cell population, relative to the vehicle control growth, over the entire expression period and prior to mutant selection. In general, highly toxic treatments will reduce the growth rate as well as the survival.

Values less than 100% indicate growth inhibition. For example, 50% and 25% relative growth values would indicate treated cell populations that were one and two population doublings behind the negative control culture. Treated populations that are more than two or three doublings behind the control might not achieve maximum expression of the TG-resistant phenotype. The relative population growth is calculated from cell count data not presented in this report and is intended to provide only an approximate indication of growth during the expression period, since cells are easily lost or not completely released by trypsin during the subculture procedures.

$$\text{Relative Population Growth (\%)} = \frac{\text{Treated culture population increase over the expression period}}{\text{Vehicle control population increase over the expression period}} \times 100\%$$

Absolute Cloning Efficiency: The ability of the cells to form colonies at the time of mutant selection is measured by the absolute cloning efficiency (CE). This parameter is used as the best estimate of the cloning efficiency of the mutant cells in the selection dishes. Thus, the observed number of mutant colonies can be converted to the frequency of mutant cells in the treated population.

$$\text{Absolute CE (\%)} = \frac{\text{Average no. of viable colonies per dish}}{200} \times 100\%$$

Mutant Frequency

The mutant frequency is the endpoint of the assay. It is calculated as the ratio of colonies found in thioguanine- selection medium to the total number of cells seeded, adjusted by the absolute C.E. The frequency is expressed in units of 10^{-6}, e.g., the number of mutants per one million cells.

$$\text{Mutant Frequency} = \frac{\text{Total mutant clones}}{\text{no. of dishes} \times 2 \times 10^5 \times \text{abs. C.E}}$$

Assay Acceptance Criteria

An assay normally is considered acceptable for evaluation of the results only if all of the following criteria are satisfied. The activation and nonactivation portions of the mutation assay may be performed concurrently, but each portion is, in fact, an independent assay with its own positive and vehicle controls. The activation or nonactivation assays will be repeated independently, as needed, to satisfy the acceptance and evaluation criteria.

- The average absolute cloning efficiency of the vehicle controls should be between 70% and 115%. A value greater than 100% is possible because of errors in cell counts (usually ± 10%) and dilutions during cloning. Cloning efficiencies below 70% do not necessarily indicate substandard culture conditions or unhealthy cells. Assay variables can lead to artificially low cloning efficiencies in the range of 50 to 70% and still yield internally consistent and valid results. Assays with cloning efficiencies in this range will be conditionally acceptable and dependent on the scientific judgment of the Study Director. All assays below 50% cloning efficiency will be unacceptable.

- The background mutant frequency (average of the vehicle controls) is calculated separately for the activation and nonactivation assays, even though the same population of cells may be used for concurrent assays. The activation vehicle controls contain the S9 activation mix and may have a slightly different mutant frequency than the nonactivation vehicle controls. For both conditions, background frequencies for assays performed with different cell stocks are generally 0 to 10×10^{-6}. Assays with backgrounds greater than 15×10^{-6} will not be used for evaluation of a test article.

- A positive control is included with each assay to provide confidence in the procedures used to detect mutagenic activity. An assay will be acceptable in the absence of a positive control (loss due to contamination or technical error) only if the test article clearly shows mutagenic activity as described in the evaluation criteria. If the test article appears to have no or only weak mutagenic activity, an acceptable assay must have a positive control mutant frequency that is significantly elevated over the concurrent vehicle controls ($p \leq 0.01$).

- For test articles with little or no mutagenic activity, an acceptable assay should include applied concentrations that reduce the clonal survival to approximately 10% to 15% of the average of the vehicle controls, reach the maximum applied concentrations given in the evaluation criteria, reach a concentration that is approximately twice the solubility limit of the test article

in culture medium or include a high concentration that is at least 75% of an excessively toxic concentration. There is no maximum toxicity requirement for test articles which clearly show mutagenic activity.

- Mutant frequencies are normally derived from sets of twelve dishes for the mutant colony count and three dishes for the viable colony count. To allow for contamination losses, an acceptable mutant frequency for treated cultures can be calculated from a minimum of eight mutant selection dishes and two cloning efficiency dishes.

- The mutant frequencies for five treated cultures are normally determined in each assay. A required number of different concentrations cannot be explicitly stated, although a minimum of three analyzed cultures is considered necessary under the most favorable test conditions in order to accept a single assay for evaluation of the test article.

Assay Evaluation Criteria

Mutation assays are initiated by exposing cell cultures to about six to eight concentrations of test article that are expected, on the basis of preliminary toxicity studies, to span a range of cellular responses from no observed toxicity to about 10% survival. Usually five doses are selected for completion of the mutation assay. These doses should cover a range of toxicities with emphasis placed on the most toxic doses. An assay may need to be repeated with different concentrations to properly valuate a test article.

The statistical tables provided by Kastenbaum and Bowman (1970) are used to determine whether the results at each dose are significantly different from the negative controls at 95% or 99% confidence levels. This test compares variables distributed according to Poissonian expectations by summing up the probabilities in the tails of two binomial distributions. The 95% confidence level must be met as one criterion for considering the test article to be active at a particular dose. In addition, the mutant frequency must meet or exceed 15×10^{-6} in order to compensate for random fluctuations in the 0 to 10×10^{-6} background mutant frequencies that are typical for this assay.

Observation of a mutant frequency that meets the minimum criteria for a positive response in a single treated culture within a range of assayed concentrations is not sufficient evidence to evaluate a test article as a mutagen. The following test results must be obtained to reach this conclusion for either activation or non-activation conditions:

- A dose-related or toxicity-related increase in mutant frequency should be observed. It is desirable to obtain this relation for at least three doses. However, this depends on the concentration steps chosen for the assay and the toxicity at which mutagenic activity appears. If an increase in mutant frequency is observed for a single dose near the highest testable

toxicity, as defined previously, and the number of mutant colonies is more than twice the value needed to indicate a significant response, the test article generally will be considered mutagenic. Smaller increases at a single dose near the highest testable toxicity will require confirmation by a repeat assay.

- For some test articles, the correlation between toxicity and applied concentration is poor. The proportion of the applied article that effectively interacts with the cells to cause genetic alterations is not always repeatable or under control. Conversely, measurable changes in the frequency of induced mutants may occur with concentration changes that cause only small changes in observable toxicity. Therefore, either parameter, applied concentration or toxicity (percent survival), can be used to establish whether the mutagenic activity is related to an increase in effective treatment.

A test article is evaluated as nonmutagenic in a single assay only if the minimum increase in mutant frequency is not observed for a range of applied concentrations that extends to concentrations causing about 10 % to 15 % survival or extends to a concentration at least 75 % of that causing excessive toxicity. If the test article is relatively nontoxic, the maximum applied concentrations will normally be 5 mg/mL (or 5 µL/mL) for water-soluble materials or 1 mg/mL (or 1 µL/mL) for materials in organic solvents. If a repeat assay does not confirm an earlier, minimal response as discussed above, the test article is evaluated as nonmutagenic in this assay system.

This presentation may not encompass all test situations, and the Study Director may use other criteria to arrive at a conclusion, especially when data from several repeat assays are available. The interpretation of the results in the Results and Discussion section provides the reasoning involved when departures from the above descriptions occur.

RESULTS AND DISCUSSION

Test Material Handling

The test material, CPFB, was found to be soluble in dimethylsulfoxide (DMSO) and tetrahydrofuran at about 100 mg/mL. The 100 mg/mL stock solutions were diluted 1:10 into culture medium. Immiscible globules of test material formed at 10 mg/mL settled to the bottom of the glass vial before coalescing into larger globules. Similar results were obtained when the test article was diluted directly 1:10 into culture medium. DMSO and tetrahydrofuran were not suitable solvents for preparing treatment media.

In order to achieve as uniform an exposure as possible for cell monolayers treated with the test material in culture medium, Pluronic® F-68 was investigated as an emulsifying agent for the test material. The surfactant Pluronic® F-68, manufactured by BASF Wyandotte, was used to prepare

emulsions of the sample for use as 10X primary stocks. The Pluronic® F-68 was prepared at 10% w/v in sterile deionized water followed by filtration through a 0.45 µm filter. The initial stock for each assay was prepared by adding together the desired weight of test material and volume of Pluronic® F-68. Stable emulsions were produced after this preparation was homogenized using a Tissumizer®. Lower 10X stocks were prepared by dilution with 10% Pluronic® F-68 solution.

Rangefinding Cytotoxicity Assay

The sample, CPFB, was tested in the preliminary rangefinding cytotoxicity assay with and without S9 metabolic activation. Ten doses were used in each case that ranged from 0.005 to 5.0 mg/mL.

The rangefinding cytotoxicity assay showed that the test material was toxic to CHO cells in culture both with and without S9 metabolic activation (Tables 3-1 and 3-2). Without activation, the test material was nontoxic from 0.005 to 0.02 mg/mL, followed by increased toxicity at 0.05 mg/mL. Total cell killing was obtained at 0.5 mg/mL and higher. With activation, no toxicity was observed from 0.005 to 0.2 mg/mL. Total, or near-total, cell killing was obtained at 0.5 mg/mL and higher. The results from the preliminary rangefinding cytotoxicity assays were used to select doses for the mutation assays. Treatment conditions chosen for both the nonactivation and S9 metabolic activation portions of the mutation assay covered a 100-fold range from 0.01 to 1.0 mg/mL.

Mutation Assay Without Metabolic Activation

Under nonactivation test conditions, the cultures treated with CPFB showed a dose-related decrease in both relative survival and relative population growth (Table 3-3). The cultures exposed to 0.4 mg/mL and 1.0 mg/mL were completely toxic and were terminated. An intermediate dose, 0.5 mg/mL, had less than ten percent relative clonal survival. Seven doses were available for analysis.

Without S9 metabolic activation, the mutant frequency of cultures treated with the test material varied within the acceptable range of vehicle control mutant frequency variation which is 0 to 15 $\times$ 10^{-6}. There was no positive correlation of mutant frequency with dose and no treated culture had a mutant frequency that was both significantly elevated over the average background mutant frequency of the concurrent vehicle controls and higher than the acceptable range of background mutant frequencies. One culture did have a mutant frequency less than 15 $\times$ 10^{-6} that was significantly elevated over the mutant frequencies of the concurrent vehicle controls. The mutant frequency of this single culture appeared to represent normal assay variation. Therefore, CPFB was evaluated as negative for inducing forward mutations at the HGPRT locus in CHO cells in the absence of S9 metabolic activation.

The positive control treatment with 50 µg/mL 5-bromo-2'-deoxyuridine induced a large, significant ($p \leq 0.01$) increase in mutant frequency. The background mutant frequencies of the two vehicle controls were acceptable. The assay results achieved all assay acceptance criteria, which provided confidence in the assumption that the recorded data represented a typical response of the test material in the nonactivation assay system.

Mutation Assay With Metabolic Activation

Two independent mutation assays were performed with the test material using activation conditions (Tables 3-4 and 3-5). In each trial the test material produced dose-related toxicity. In the first trial, 10 doses were used that ranged from 0.01 to 1.0 mg/mL (Table 3-4). The test material had less than two percent relative clonal survival at 0.75 mg/mL and 1.0 mg/mL. The 1.0 mg/mL culture was terminated at the time of plating for mutant selection, while the 0.75 mg/mL culture was plated for mutant selection, but due to the low survival, was not used in the evaluation of the data. The remaining eight doses showed a clonal survival range relative to the concurrent vehicle controls of 96.6 to 14.3%. As shown in Table 3-4, mutant frequencies of treated cultures were within the acceptable range for background mutant frequency variation of between 0 and 15 $\times$ 10^{-6} with the exception of the culture treated with 0.5 mg/mL. That culture had a mutant frequency of 17.4 $\times$ 10^{-6} that was also statistically elevated over the mutant frequencies of the concurrent vehicle control cultures. The mutant frequencies of the other cultures varied randomly with dose and no other culture had a significantly elevated mutant frequency. Assay evaluation criteria required a second mutation assay to properly evaluate the elevated mutant frequency at the highest acceptable dose.

The second S9 metabolic activation mutation assay used a modified dose range to focus on the toxic range where significance occurred in the first trial. Six doses were used that ranged from 0.1 to 0.6 mg/mL (Table 3-5). The six doses analyzed had relative survivals that ranged from 103.2% at 0.1 mg/mL to 12.9% for the culture at 0.5 mg/mL. For unknown reasons the culture treated with the highest dose, 0.6 mg/mL, was less toxic than the next lower dose and had 67.5 percent relative clonal survival. The mutant frequencies of all the treated cultures varied randomly with dose and were within the range for acceptable background mutant frequencies. The mutant frequency of one of the six cultures was significantly elevated over the mutant frequencies of the concurrent vehicle control cultures.

The mutant frequencies of cultures treated with CPFB were within the acceptable background range with one exception. While the pattern of mutant frequencies in the first trial was suggestive of a dose response, this pattern was not confirmed in the second trial. The significant mutant frequency seen at 0.5 mg/mL in the first trial was not confirmed in the same dose in trial II. The significance observed was apparently the result of normal assay variation. Therefore, CPFB was evaluated as

negative for inducing forward mutations at the HGPRT locus in CHO cells under the S9 metabolic activation conditions used in the study.

The positive control treatment with 5 µg/mL 3-methylcholanthrene induced a large, significant ($p \leq 0.01$) increase in mutant frequency which demonstrated the effectiveness of the S9 metabolic activation system and the ability of the test system to detect known mutagens. The background mutant frequencies of the negative control and the 1% Pluronic F-68® vehicle controls were within the acceptable range and comparable to the historical activation control data (see Appendix 3-A). The assay results achieved all assay acceptance criteria and provided confidence in the assumption that the recorded data represented typical responses of the test material in the assay system.

CONCLUSION

The test material, CPFB, is considered negative for inducing forward mutations at the HGPRT locus in CHO cells under both the S9 metabolic activation and nonactivation conditions of the assay.

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TABLE 3-1. CLONAL CYTOTOXICITY ASSAY OF CPFB WITHOUT METABOLIC ACTIVATION

Applied Concentration mg/mL	Number of Colonies	Relative Survival[a] (Percent)	Cloning Efficiency (Percent)
NC[b]	187	90.3	
VC, 1%[c]	207	100.0	103.5
Test Material			
0.005	197	95.2	
0.01	180	87.0	
0.02	189	91.3	
0.05	116	56.0	
0.1	152	73.4	
0.2	124	59.9	
0.5	0	0.0	
0.1	0	0.0	
2.0	0	0.0	
5.0	0	0.0	

[a]Relative to vehicle control
[b]NC = negative control, F12 medium
[c]VC = vehicle control, 1% Pluronic® F-68

TABLE 3-2. CLONAL CYTOTOXICITY ASSAY OF CPFB WITH S9 METABOLIC ACTIVATION

Applied Concentration mg/mL	Number of Colonies	Relative Survival[a] (Percent)	Cloning Efficiency (Percent)
NC[b]	189	118.1	
VC, 1%[c]	160	100.0	80.0
Test Material			
0.005	183	114.4	
0.01	154	96.3	
0.02	170	106.3	
0.05	159	99.4	
0.1	208	130.0	
0.2	192	120.0	
0.5	5	3.1	
1.0	0	0.0	
2.0	0	0.0	
5.0	0	0.0	

[a]Relative to vehicle control.
[b]NC = Negative control, F12 medium
[c]VC = Vehicle Ccontrol 1% Pluronic® F-68 .

TABLE 3-3. MUTATION ASSAY OF CPFB WITHOUT METABOLIC ACTIVATION - TRIAL I

NON-ACTIVATION TEST CONDITION	SURVIVAL TO TREATMENT % VEH. CONTROL	RELATIVE POPULATION GROWTH (% OF CONTROL)	TOTAL MUTANT COLONIES	ABSOLUTE C.E. ± S.D. (%)	MUTANT FREQ IN 10^{-6} UNITS[a]
Vehicle Control[b]	95.9	92.2	15	103.0 ± 4.6	6.1
Vehicle Control[b]	104.1	107.8	15	84.0 ± 1.8	7.4
Positive Control (50 µg/mL BrdU)[c]	71.6	43.0	228	90.2 ± 2.1	105.3[d]
CPFB(mg/mL)					
0.01	90.9	82.0	19	92.5 ± 1.3	8.6
0.02	90.2	81.1	19	93.5 ± 4.3	8.5
0.05	83.6	83.6	26	102.7 ± 1.1	10.5
0.1	92.7	88.1	17	90.0 ± 9.3	7.9
0.2	59.4	75.5	31	100.7 ± 5.5	12.8[e]
0.3	22.0	18.9	7	98.0 ± 6.3	3.0
0.4	0.0	T[f]	–	–	–
0.5	7.5	34.1	11	92.4 ± 0.8	5.0
1.0	0.0	T	–	–	–

[a] Mutant Frequency = Total mutant colonies/ (No. of dishes x 2 x 10^5 x absolute C.E.)
[b] Vehicle Control = 1% Pluronic® F-68
[c] Brdu = 5-Bromo-2'-deoxyuridine
[d] Significant increase: Kastenbaum Bowman test $p \leq 0.01$ and mutant frequency $\geq 15 \times 10^{-6}$
[e] Kastenbaum-Bowman test $p \leq 0.01$ but mutant frequency is within acceptable background range ($< 15.0 \times 10^{-6}$).
[f] T = Terminated due to excessive toxicity

TABLE 3-4. MUTATION ASSAY OF CPFB WITH METABOLIC ACTIVATION - TRIAL I

ACTIVATION TEST CONDITION	SURVIVAL TO TREATMENT % VEH. CONTROL	RELATIVE POPULATION GROWTH (% OF CONTROL)	TOTAL MUTANT COLONIES	ABSOLUTE C.E. ± S.D. (%)	MUTANT FREQ IN 10^{-6} UNITS[a]
Negative Control[b]	98.0	109.3	7	100.4 ± 4.6	2.9
Vehicle Control[b]	104.7	103.6	11	97.0 ± 4.1	4.7
Vehicle Control[b]	95.3	96.4	5	103.0 ± 8.2	2.0
Positive Control (5 µg/mL 3-MCA)[c]	82.3	60.8	715	105.0 ± 6.9	283.7[d]
CPFB(mg/mL)					
0.01	96.6	76.9	14	115.9 ± 7.3	5.0
0.05	86.3	NS[e]	-	-	-
0.1	88.1	65.4	3	107.7 ± 8.3	1.2
0.2	69.8	57.0	16	107.4 ± 3.9	6.2
0.3	31.7	25.8	8	101.4 ± 9.5	3.3
0.4	37.2	37.4	7	96.7 ± 6.0	3.0
0.45	25.0	20.8	13	92.9 ± 1.3	5.8
0.5	14.3	15.1	43	102.7 ± 6.1	17.4[d]
0.75	1.2	6.4	3	81.4 ± 4.3	1.5
1.0	0.9	T[f]	-	-	-

[a] Mutant Frequency = Total mutant colonies/(No. of dishes × 2 × 10^5 × absolute C.E.)
[b] Negative Control - F12 culture medium, Vehicle Control = 1% Pluronic® F-68
[c] 3-MCA = 3-Methylcholanthrene
[d] Significant increase: Kastenbaum Bowman test $p \leq 0.01$ and mutant frequency $\geq 15.0 \times 10^{-6}$
[e] NS = Not plated for selection due to sufficient surviving higher dose levels
[f] T = Terminated due to excessive toxicity

TABLE 3-5. MUTATION ASSAY OF CPFB WITH METABOLIC ACTIVATION - TRIAL II

ACTIVATION TEST CONDITION	SURVIVAL TO TREATMENT % VEH. CONTROL	RELATIVE POPULATION GROWTH (% OF CONTROL)	TOTAL MUTANT COLONIES	ABSOLUTE C.E. ± S.D. (%)	MUTANT FREQ IN 10^{-6} UNITS[a]
Vehicle Control[b]	97 5	100 9	12	85 2 ± 0 8	5 9
Vehicle Control[b]	102.5	99 1	9	86.7 ± 2 8	4 3
Positive Control (5 µg/mL 3-MCA)[c]	98 7	71 5	660	82.7 ± 11.6	332 5[d]
CPFB (mg/mL)					
0.1	103 2	105 8	14	87.9 ± 7.3	6 6
0.3	37.1	58 2	14	83 5 ± 5 4	7 0
0.4	30.5	66 9	26	82 9 ± 6 9	13 1[e]
0 45	18.7	41 2	17	88.7 ± 6 3	8 0
0.5	12.9	19 6	8	84 9 ± 2 9	3 9
0 6	67 5	66 1	11	83 0 ± 2 2	5 5

[a] Mutant Frequency = Total mutant colonies/(No of dishes × 2×10^5 × absolute C E)
[b] Vehicle Control = 1% Pluronic® F-68
[c] 3-MCA = 3-Methylcholanthrene
[d] Significant increase Kastenbaum Bowman test $p \leq 0.01$ and mutant frequency $\geq 15.0 \times 10^{-6}$
[e] Kastenbaum-Bowman test $p \leq 0.01$ but mutant frequency is within acceptable background range ($.15.0 \times 10^{-6}$)

APPENDIX 3-A

HISTORICAL CHO HGPRT ASSAY CONTROL MUTANT FREQUENCY DATA

A. Nonactivation Studies

1. Pooled negative and solvent controls

Mean (± SD)	$3.9 \pm 2.9 \times 10^{-6}$
Range	0 to 16.8×10^{-6}
Number of experiments	50
Number of controls	88

2. Positive controls (50 µg/mL 5-bromo-2'-deoxyuridine)

Mean (± SD)	$121.6 \pm 27.9 \times 10^{-6}$
Range	38.7 to 165.6×10^{-6}
Number of experiments	50
Number of controls	59

B. Activation Studies

1. Pooled negative and solvent controls

Mean (± SD)	$2.9 \pm 2.1 \times 10^{-6}$
Range	0 to 10.0×10^{-6}
Number of experiments	50
Number of controls	86

2. Positive controls (5 µg/mL 3-methylcholanthrene)

Mean (± SD)	$370.0 \pm 173.3 \times 10^{-6}$
Range	152.3 to 941.6×10^{-6}
Number of experiments	50
Number of controls	61

The historical control data was compiled from the most recent fifty experiments. Because some experiments contained duplicate controls, the number of independent control cultures exceeded the number of experiments.

SECTION 4

MUTAGENICITY TEST ON CHLOROPENTAFLUOROBENZENE IN AN *IN VITRO* CYTOGENETIC ASSAY MEASURING SISTER CHROMATID EXCHANGE AND CHROMOSOMAL ABERRATION FREQUENCIES IN CHINESE HAMSTER OVARY CELLS

Murli, H.[a]

ABSTRACT

The objective of this *in vitro* assay was to evaluate the ability of chloropentafluorobenzene (CPFB) to induce sister chromatid exchange (SCE) and chromosomal aberrations in Chinese hamster ovary (CHO) cells with and without metabolic activation. In the SCE assay single cultures of CHO cells were incubated with 0.167 to 5010 µg/mL in a half-log series. There was no significant increase in SCE at the concentrations tested in the nonactivation assay. A significant increase in SCE was seen at 16.7, 50.1; and 167 µg/mL in the activation assay. The activation SCE assay was repeated to verify the positive response testing concentrations of 50.1 to 501 µg/mL. A significant increase in SCE was again observed at all the concentrations analyzed.

Based on the evaluation of cell cycle kinetics from the SCE assay, a 10 h harvest was conducted testing concentrations of 20 to 200 µg/mL, and a 20 h harvest was conducted testing concentrations of 200 to 500 µg/mL in the nonactivation aberrations assay. A 10 h harvest was conducted testing concentrations of 50 to 500 µg/mL in the aberrations assay with activation. No increase in chromosomally aberrant cells was observed at any of the concentrations analyzed.

The test article, CPFB, is considered negative for inducing SCE under nonactivation conditions but positive for inducing SCE under conditions of activation. CPFB is considered negative for inducing chromosomal aberrations in CHO cells under both nonactivation and activation conditions of this assay.

INTRODUCTION

Sister chromatid exchanges (SCE) are seen at metaphase as reciprocal interchanges of the two chromatid arms within a single chromosome. These exchanges presumably require enzymatic incision, translocation, and ligation of the two DNA strands. The frequency of SCE is thought to be a very sensitive indicator of damage to the genetic material, DNA. Increases in the frequency of SCE are caused by many chemical agents known to be mutagens/carcinogens. Thus SCE test is a relevant cytogenetic test for potentially genotoxic chemicals.

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The SCE test involves treating cultured cells with a test compound, growing cells with the thymidine analog 5-bromo-2'-deoxyuridine (BrdU) for ~2 cell cycles, and making chromosome preparations that are stained for SCE. The chromosomes of dividing cells consist of two identical halves, or sister chromatids. By growing cells with BrdU for two cell cycles one chromatid contains half as much BrdUrd as the other and is stained more intensely by Giemsa, while its pair, or sister, is pale.

This assay is also designed to establish whether the test article or its metabolites can interact with cells to induce chromosomal breaks. Chemically induced lesions may result in breaks in chromatin that are either repaired by the cell in such a way as to be undetectable or result in visible damage. Aberrations are a sequence of failure or mistakes in repair processes such that breaks do not rejoin or rejoin in abnormal configurations (Evans, 1962).

The chromosomal aberrations assay is designed to examine cells in the first mitosis after chemical exposure. This design limits loss of aberrant cells during the division process or conversion into complex derivatives during subsequent cell cycles. In the case of CHO cells most dividing cells examined 8-12 h after treatment are in the first mitosis (M_1 cells). However, many test articles cause severe delay of progression through the cell cycle, and the assay has been designed to detect this delay and allow for slower growth of damaged cells by adjustments in the time between treatment and cell fixation.

The objective of this in vitro assay was to evaluate the ability of CPFB to induce SCE and chromosomal aberrations in CHO cells, with and without metabolic activation.

EXPERIMENTAL DESIGN

In the SCE assay, CHO cell cultures which were exposed to the test article for approximately two cell cycles were analyzed to determine cellular toxicity and effects of the test article on cell generation time. If necessary and possible, the assay was extended in cultures at affected doses to allow for the progression to second generation cells. The doses used in the assay ranged from 0.167 µg/mL of the test article solution through 5010 µg/mL in a half-log series. Single cultures were used for the negative control, solvent control, each of two doses of the positive control and ten doses of the test material. Sister chromatid exchange frequencies were analyzed from cultures treated with the four highest doses with second generation cells and from a negative, solvent and positive control culture. Cell cycle kinetics of the treated cultures were also evaluated.

SUMMARY OF SCE ASSAY TREATMENT SCHEDULE IN HOURS

Test	Test Article	Wash	BrdU	Wash	Colcemid®	Fixation
-S9	-2.25		0	22.75	23	25.5
+ S9	-2.25	-0.25	0		23	25.5

Cell cycle kinetics from the SCE assay were used 1) to determine the dose range to be used in the chromosomal aberrations assay and 2) to determine the optimal time of harvest of the dosed cells so that primarily metaphase cells which were in the first metaphase since exposure to the test article would be analyzed for chromosomal aberrations. The aberrations assay was conducted at the 10 h harvest time for those chemicals which did not induce any cell cycle delay and at the 20 h harvest time for those chemicals that induced cell cycle delay.

In the chromosomal aberrations assays duplicate cultures were used at each dose. Single cultures were used for the negative control, solvent control, and at each of two doses of the positive control. In the nonactivation assay, 10 and 20 h harvests were conducted. In the activation assay, 10 h harvests were conducted. Chromosomal aberrations were analyzed from the four highest doses from which results could be obtained and from only one of the positive control doses. A summary of the treatment schedule for the chromosomal aberrations assays is given below.

SUMMARY OF CHROMOSOMAL ABERRATIONS ASSAY TREATMENT SCHEDULE IN HOURS

Test	Test Article	Wash	Colcemid®	Fixation
-S9	0	7.25	7.5	10
-S9	0	17.25	17.5	20
+ S9	0	2	7.5	10

MATERIALS AND METHODS

Test Material:

Chloropentafluorobenzene 99 + %

Lot # 01721MV

Clear colorless liquid

Indicator Cells

The Chinese hamster ovary cells (CHO-WBL) used in this assay were from a permanent cell line and were originally obtained from the laboratory of Dr. S. Wolff, University of California, San Francisco. The cells have since been recloned to maintain karyotypic stability. This cell line has an average cycle time of 12 to 14 h with a modal chromosome number of 21.

Cell Culture Medium

The CHO cells were grown in McCoy's 5a culture medium which was supplemented with 10% fetal calf serum (FCS), 1% L-glutamine, and 1% penicillin and streptomycin, at about 37°C, in an atmosphere of about 5% CO_2 in air.

Negative and Solvent Controls

In the nonactivation assays, negative controls were cultures which contain only cells and culture medium. Solvent controls were cultures containing the solvent for the test article at the same concentration used in test cultures. In the activation assays, the negative and solvent controls were as in the nonactivation assays but the S9 activation mix was also added.

Positive Control Agents

The positive control agents which were used in the assays were mitomycin C (MMC) for the nonactivation series and cyclophosphamide (CP) in the metabolic activation series. Mitomycin C is a clastogen that does not require metabolic activation. Cyclophosphamide does not act directly but must be converted to active intermediates by microsomal enzymes. In the SCE assay two doses of MMC (0.005 µg/mL and 0.010 µg/mL) and CP (1.50 µg/mL and 2.00 µg/mL) were used. In the chromosomal aberrations assays two concentrations of MMC (0.500 and 1.00 µg/mL, 10 h harvest; 0.040 and 0.080 µg/mL, 20 h harvest) and CP (25.0 µg/mL and 50.0 µg/mL) were used to induce chromosomal aberrations in the CHO cells. Only one dose of the positive control was actually analyzed in each of the SCE and aberration assays.

Sister Chromatid Exchange Assays

In these assays, the cells were cultured for approximately 24 h prior to treatment by seeding approximately 0.8×10^6 cells per 75 cm^2 flask into 10 mL of complete McCoy's 5a culture medium. The thymidine analog, BrdUrd, was added at a final concentration of 10 µM approximately two hours after the initial exposure of the cells to the test article.

Nonactivation Assay

The cultures were dosed with the test article for ~two hours when (BrdU) was added at a final concentration of 10 µM. The cultures were then reincubated for approximately 23 h.

Approximately 2.75 h prior to the harvest of the cells, the test article was washed from the cells with phosphate buffered saline and fresh complete medium with BrdU (10 µM), and Colcemid® (final concentration 0.1 µg/mL) was added.

Prior to the harvest of the cultures visual observations of toxicity were made. These observations included an assessment of the percent confluence of the cell monolayer within the culture flasks. The cultures were also evaluated for the presence of mitotic (large rounded cells) or dead cells floating in the medium. Only flasks from the highest five surviving doses from which metaphase cells for analysis were expected were harvested (See Section on Harvest). Medium was collected and the cultures centrifuged. The mitotic cells were fixed but medium was replaced on the remaining cell monolayer in the flasks. A test slide was made from fixed cells treated with the highest doses of test compound, and stained with Hoechst 33258 stain (0.5 µg/mL in phosphate buffer, pH 6.8). The slides were examined under UV fluorescence microscopy As there was no marked cell cycle delay, a second cell collection was not made. The harvested cells were differentially stained for the analysis of SCE using a modified fluorescence-plus-Giemsa (FPG) technique (See Sections on Harvest and Slide Preparation and staining).

Assay with Metabolic Activation

In this assay, the CHO cells were exposed to the test article for two hours in the presence of a rat liver S9 reaction mixture (S9 15 µL/mL, NADP 1.5 mg/mL, and isocitric acid 2.7 mg/mL). The S9 fraction was derived from the liver of male Sprague-Dawley rats which had been previously treated with Aroclor 1254 to induce the mixed function oxidase enzymes which are capable of metabolizing chemicals to more active forms. The two hour incubation time was used because prolonged exposure to the S9 mixture might be toxic to the cells and the enzyme activity of S9 is lost rapidly at 37°C. The medium did not have FCS during the exposure period to avoid possible inactivation of short-lived and highly reactive intermediates produced by the S9 enzymes by binding to serum proteins.

In this assay, the CHO cells were incubated at 37°C for two hours in the presence of the test article and the S9 reaction mixture in the growth medium without FCS. After the exposure period the cells were washed twice with buffered saline. Complete McCoy's 5a medium with 10 µM BrdU was added to the cultures which were then incubated for approximately 23 h. Colcemid® (final concentration 0.1 µg/mL) was then added and the cultures were then reincubated for 2.5 h, harvested and examined for any cell delay. Slides were prepared and stained as described for the nonactivation assay. Delayed fixation was not required for these cultures.

Nonactivation Aberrations Assays

Cultures were initiated by seeding approximately 1.0 x 10^6 cells (20 hour assay) and 1.5 x 10^6 cells (10 h assay) per 75 cm^2 flask into 10 mL of complete McCoy's 5a medium. One day after culture initiation, the CHO cells to be used in the nonactivation trial were treated with the test article at predetermined doses for 7.25 and 17.25 h. The cultures were then washed with buffered saline and complete McCoy's 5a medium containing 0.1 µg/mL Colcemid® was placed back onto the cells. Two and one half hours later the cells were harvested and air dried slides were made. The slides were then stained in pH 6.8 buffered 5% Giemsa solution for the analysis of chromosomal aberrations.

Aberrations Assays with Metabolic Activation

Cultures were initiated by seeding approximately 1.5 x 10^6 cells per 75 cm^2 flask into 10 mL of complete McCoy's 5a medium. One day after culture initiation, the cultures that were treated under the conditions of metabolic activation were incubated at 37°C for two hours in the presence of the test article and the S9 reaction mixture in McCoy's 5a medium without FCS. After the two hour exposure period the cells were washed twice with buffered saline and the cells were refed with complete McCoy's 5a medium. The cells were incubated for an additional 7.5 h with 0.1 µg/mL Colcemid® present during the last 2.5 h of incubation. The metaphase cells were then harvested and prepared for cytogenetic analysis.

Harvest Procedure

Prior to the harvest of the cultures visual observations of toxicity were made. These observations included an assessment of the percent confluence of the cell monolayer within the culture flasks. The cultures were also evaluated for the presence of mitotic (large rounded cells) or dead cells floating in the medium. The metaphase cells were collected by mitotic shake-off (Terasima and Tolmach, 1961) and were treated with 0.075 M KCl solution. This treatment helps to swell the cells and thus disperse the chromosomes. The cultures were then fixed with an absolute methanol: glacial acetic acid (3:1) fixative and were washed several times before air-dried slides were prepared.

Slide Preparation and Staining

Slides were prepared by dropping the harvested cultures on clean slides. The slides from the rangefinding assays were differentially stained using a modified fluorescence-plus-Giemsa (FPG) technique (after Perry and Wolff, 1974; Goto, et al., 1978). The slides were stained in Hoechst 33258 stain, exposed to ultraviolet light, and then stained with Giemsa Azure B stain. The slides prepared from the aberrations assay were stained with pH 6.8 buffered 5% Giemsa solution for the analysis of chromosomal aberrations. All slides were then air-dried and coverslipped using Depex® mounting medium.

SCE Analysis and Assay Evaluation

Fifty cells per dose were analyzed from each of the top four doses from which sufficient M_2 metaphase cells were available. Fifty cells were read from each of the negative and solvent controls, and at least twenty cells were read from one dose level of the positive control. For control of bias, all slides except for the positive controls were coded prior to analysis. Cells were selected for scoring on the basis of good morphology and clear sister chromatid differentiation along the entire length of all chromosomes; only cells with the number of centromeres equal to the modal number 21 ± 2 (range of 19-23) were analyzed.

The slides were examined for the presence of delayed cells. One hundred metaphase cells were scanned and classified as M_1, M_1+, or M_2 from each dose and the positive, negative, and solvent controls to give an estimate of cell cycle inhibition. In those doses where more than one harvest was carried out, cells were analyzed for cell cycle kinetics and SCE from the earliest harvest time from which sufficient M_2 cells were available for analysis. Controls were analyzed only at the normal harvest time (25-26 h).

Data were collected on standard forms. The data were summarized in tables showing the numbers of cells scored, total SCEs, SCE per chromosome, and SCE per cell. The cell cycle kinetics were also calculated.

If an increase in SCE was observed, one of the following criteria must normally be met to assess the compound as positive:

> *Two-fold increase:* Approximately a doubling in SCE frequency over the "background" (solvent and negative control) levels at one or more doses.
>
> *Dose response:* A positive assessment may be made in the absence of a doubling if there was a statistically significant increase at a minimum of three doses and evidence for a positive dose response.

Statistical analysis employed a Student t-test (Bancroft, 1957; Hollander and Wolfe, 1973) to compare SCE frequencies in the tested cultures with the negative and solvent controls. The final evaluation of the test article was based upon scientific judgment.

Aberrations Analysis and Assay Evaluation

Cells were selected for good morphology and only cells with the number of centromeres equal to the modal number 21 ± 2 (range 19-23) were analyzed.

One hundred cells, if possible, from each duplicate culture at four (10 h assays) and one (20 h nonactivation assay) doses of the test article and from each of the negative and solvent control cultures were analyzed for the different types of chromosomal aberrations (Evans, 1962; See

Appendix 1). At least 25 cells were analyzed for chromosomal aberrations from one of the positive control cultures. For control of bias, all slides except for the positive controls were coded prior to analysis. Cells with aberrations were recorded on the data sheets by the microscope stage location.

The following factors were taken into account in the evaluation of the chromosomal aberrations data:

The overall chromosomal aberration frequencies.

The percentage of cells with any aberrations.

The percentage of cells with more than one aberration.

Any evidence for increasing amounts of damage with increasing dose, i.e., a positive dose response.

The estimated number of breaks involved in the production of the different types of aberrations which were observed, i.e., complex aberrations may have more significance than simple breaks.

Chromatid and isochromatid gaps, if observed, were noted in the raw data and were tabulated. They were not, however, considered in the evaluation of the ability of the test article to induce chromosomal aberrations since they may not represent true chromosomal breaks and may possibly be induced by toxicity.

A cell classified as "GT" is considered to contain 10 aberrations for statistical purposes but a ">" is also included in the tables for this classification to indicate that it is a minimum number.

Statistical analysis employed the Fisher's Exact Test with an adjustment for multiple comparisons (Sokal and Rohlf, 1981) to compare the percentage of cells with aberrations in each treatment group with the results from the pooled solvent and negative controls (the solvent and negative controls were statistically evaluated for similarity prior to the pooled evaluation). Test article significance was established where $p \leq 0.01$. All factors as stated previously were taken into account and the final evaluation of the test article was based upon scientific judgement.

RESULTS

Solubility, Stability, and Dose Determination

Solubility of the test article was tested in dimethyl sulfoxide and tetrahydrofuran. Clear colorless solutions were obtained at a concentration of ~100 mg/mL. These solutions were diluted 1:10 in culture medium (MEM) and immiscible globules were obtained. Vortexing and shaking vigorously made the globules smaller which then settled to the bottom of the glass vial before coalescing into larger globules. Similar results were obtained when the test article was diluted directly 1:10 in culture medium. Solubility was tested further in 10% (w/v) solution of Pluronic® F-68

solution. Stable emulsions were produced after this solution was homogenized using a Tissuemizer. A top stock of 50.0 mg/mL was selected for testing in these assays. Final concentrations were achieved by a 1:10 dilution of the 50.0 mg/mL stock solution or the serial dilutions prepared from this stock solution. A half-log series of concentrations from 0.167 through 5010 µg/mL was tested in the SCE assay.

Sister Chromatid Exchange Assay Without Metabolic Activation

Dead cell monolayer and no visible mitotic cells were observed at 501, 1670, and 5010 µg/mL. No significant toxicity was observed at the subsequent concentration of 167 µg/mL. Results were evaluated at 5.01, 16.7, 50.1, and 167 µg/mL (Table 4-1). Analysis of cell cycle kinetics revealed a slight cell cycle delay at 167 µg/mL. No significant increase in SCE was observed at the concentrations analyzed, except at 16.7 µg/mL. This increase seemed significant only because of the low SCE/cell in the solvent control culture. The result at 16.7 µg/mL is not significantly greater than the negative control, or the historical solvent control results (~8.0 SCE/cell). The sensitivity of the cell culture for induction of SCE is shown by the increased frequency of SCE in the cells exposed to the positive control agent. The test article is considered negative for inducing SCE under conditions of nonactivation.

Sister Chromatid Exchange Assay With Metabolic Activation

Complete cytotoxicity was observed again at 501, 1670, and 5010 µg/mL as in the nonactivation assay. No toxicity was discernible at 167 µg/mL. Results were evaluated at 5.01, 16.7, 50.1, and 167 µg/mL (Table 4-2). A significant increase in SCE was observed at 16.7, 50.1, and 167 µg/mL. To verify the positive response this trial was repeated testing concentrations of 50.1, 100, 150, 200, 250, and 501 µg/mL. In Trial 2, complete cellular toxicity was observed at 501 µg/mL. Severe toxicity was manifested at 250 µg/mL by an unhealthy cell monolayer, floating cellular debris, few visible mitotic cells, and ~90% reduction in cell monolayer confluence. An unhealthy cell monolayer, ~50% reduction in cell monolayer confluence, floating cellular debris, and a slight reduction in visible mitotic cells were observed at 200 µg/mL. Very little toxicity was discerned at the other concentrations tested. No significant cell cycle delay was observed at these concentrations. Results were analyzed at 100, 150, 200, and 250 µg/mL (Table 4-3). A significant dose related increase was observed at all the concentrations tested. The successful activation of the metabolic system is illustrated by the increased frequency of SCE in the cells induced with the positive control agent. The test article is considered positive for inducing SCE under conditions of metabolic activation.

Chromosomal Aberrations Assay Without Metabolic Activation

Cell cycle kinetics from the nonactivation SCE assay indicated a slight cell cycle delay at 167 µg/mL. Based on this observation, two harvest times were selected for the aberrations assay. A

10 h harvest was selected for testing concentrations of 20.0, 50.1, 100, 150, and 200 µg/mL and a 20 h harvest was selected for testing concentrations of 200, 250, 300, 400, and 501 µg/mL.

In the 10 h assay, floating cellular debris, a slight reduction in visible mitotic cells, and a ~30% reduction in the cell monolayer confluence were observed at 200 µg/mL. Floating debris was observed also at 150 µg/mL. Results were evaluated at 50.1, 100, 150, and 200 µg/mL (results pooled from the replicate cultures are in Table 4-4, and results from individual cultures are in Table 4-5).

In the 20 h assay, complete cellular toxicity was observed at 250, 300, 400, and 501 µg/mL. A very unhealthy cell monolayer, severe reduction in visible mitotic cells, and ~70% reduction in the cell monolayer confluence were observed at 200 µg/mL. Results were evaluated at 200 µg/mL (pooled results from the replicate cultures are given in Table 4-6, and from individual cultures in Table 4-7).

No significant increase in chromosomally aberrant cells was observed at the concentrations analyzed with the 10 or 20 h harvest. The sensitivity of the cell culture for induction of chromosomal aberrations is shown by the increased frequency of aberrations in the cells exposed to the positive control agent. The test article is considered negative for inducing chromosomal aberrations under nonactivation conditions.

Chromosomal Aberrations Assay With Metabolic Activation

No cell cycle delay was evident in the analysis of cell cycle kinetics in the SCE assay with activation at the doses analyzed. A 10 h harvest was selected for the aberrations assay for testing concentrations of 50.1, 100, 150, 200, 250, 300, 400, and 501 µg/mL. Complete cellular toxicity was observed at 300, 400, and 501 µg/mL. Floating dead cells and cellular debris, and reduction in visible mitotic cells (more severe in one of the replicate cultures, which also showed ~50% reduction in cell monolayer confluence) were observed at 250 µg/mL. No toxicity was discerned at the other concentrations. Results were evaluated at 100, 150, 200, and 250 µg/mL (results pooled from the replicate cultures are in Table 4-8, and results from individual cultures are in Table 4-9). No significant increase in chromosomally aberrant cells was observed at the doses analyzed. The successful activation of the metabolic system is illustrated by the increased incidence of chromosomally aberrant cells in the cultures induced with cyclophosphamide, the positive control agent. The test article is considered negative for inducing chromosomal aberrations under conditions of metabolic activation.

CONCLUSION

The test article, CPFB, is considered negative for inducing SCE in CHO cells under the nonactivation conditions of this assay, but positive under conditions of metabolic activation. CPFB is considered negative for inducing chromosomal aberrations in CHO cells under both the metabolic activation and nonactivation conditions of this assay.

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TABLE 4-1. SISTER CHROMATID EXCHANGE IN CHINESE HAMSTER OVARY CELLS WITHOUT METABOLIC ACTIVATION OF CPFB

TREATMENT	DOSE µg/mL (µL/mL)	TOTAL CELLS SCORED	# OF CHROMO-SOMES	# OF SCE	SCE/ CHROMO-SOME	SCE/CELL MEAN ± S.E	TIME IN BrdU (hr)	CELL CYCLE STAGES (%) M1	M1+	M2	M2+	% SCE INCREASE OVER SOLVENT	CONFLUENCE % SOLVENT CONTROL
CONTROLS													
NEGATIVE:													
McCoys 5a		50	1037	366	0.35	7.32 ± 0.36	25.4		6.5	92.0	1.5		
SOLVENT: 10% PLURONIC®	(100)	50	1046	338	0.32	6.76 ± 0.42	25.4	0.5	7.0	92.5			100
POSITIVE: MMC	0.005	20	417	645	1.55	32.25 ± 1.54*	25.4	1.0	11.0	88.0		379	100
CPFB	5.01	50	1043	340	0.33	6.80 ± 0.42	25.4	0.5	9.5	90.0		1	100
	16.7	50	1037	405	0.39	8.10 ± 0.44*	25.4		14.0	86.0		21	100
	50.1	50	1046	373	0.36	7.46 ± 0.35	25.4	0.5	10.5	88.5	0.5	10	100
	167	50	1043	400	0.38	8.00 ± 0.37	25.4	2.0	44.5	53.5		19	100
	501**												13

*Significantly greater than the solvent control, $p<0.01$
**Toxic dose

TABLE 4-2. SISTER CHROMATID EXCHANGE IN CHINESE HAMSTER OVARY CELLS WITH METABOLIC ACTIVATION OF CPFB: TRIAL 1

TREATMENT	DOSE µg/mL (µL/mL)	TOTAL CELLS SCORED	# OF CHROMO-SOMES	# OF SCE	SCE/ CHROMO-SOME	SCE/CELL MEAN ± S E	TIME IN BrdU (hr)	CELL CYCLE STAGES (%) M1	M1+	M2	M2+	% SCE INCREASE OVER SOLVENT	CONFLUENCE % SOLVENT CONTROL
CONTROLS													
NEGATIVE													
McCoys 5a		50	1029	393	0 38	7 86 ± 0 43	25 4	0 5	6 5	92 0	1 0		
SOLVENT 10% PLURONIC®	(100)	50	1042	371	0 36	7 42 ± 0 42	25 4		3 0	90 5	6 5		100
POSITIVE CP	1 50	20	411	606	1 47	30 30 ± 1 10*	25 4	1 0	7 5	91 5		314	100
CPFB	5 01	50	1037	437	0 42	8 74 ± 0 51	25 4	0 5	4 5	93 0	2 0	18	100
	16 7	50	1045	449	0 43	8 98 ± 0 57*	25 4	0 5	3 5	91 5	4 5	21	100
	50 1	50	1045	461	0 44	9 22 ± 0 61*	25 4		8 0	89 5	2 5	24	100
	167	50	1035	553	0 53	11 06 ± 0 53*	25 4	2 5	19 0	78 5		50	100
	501**												13

*Significantly greater than the solvent control, $p<0.01$
**Toxic dose

TABLE 4-3. SISTER CHROMATID EXCHANGE IN CHINESE HAMSTER OVARY CELLS WITH METABOLIC ACTIVATION OF CPFB: TRIAL 2

TREATMENT	DOSE µg/mL (µL/mL)	TOTAL CELLS SCORED	# OF CHROMO-SOMES	# OF SCE	SCE/ CHROMO-SOME	SCE/CELL MEAN ± S.E.	TIME IN BrdU (hr)	CELL CYCLE STAGES (%) M1	M1+	M2	M2+	% SCE INCREASE OVER SOLVENT	CONFLUENCE % SOLVENT CONTROL
CONTROLS													
NEGATIVE:													
McCoys 5a		50	1046	415	0.40	8.30 ± 0.44	25.5	0.5	4.0	93.5	2.0		
SOLVENT: 10% PLURONIC®	(10)	50	1045	372	0.36	7.44 ± 0.40	25.5		3.0	93.5	3.5		100
POSITIVE: CP	1.50	20	418	1054	2.52	52.70 ± 2.98*	25.5		6.5	93.0	0.5	608	88
CPFB	100	50	1050	465	0.44	9.30 ± 0.41*	25.5	3.5	10.5	83.0	3.0	24	88
	150	50	1049	513	0.49	10.26 ± 0.54*	25.5	1.5	8.5	88.5	1.5	37	75
	200	50	1046	609	0.58	12.18 ± 0.58*	25.5	9.0	17.0	73.0	1.0	64	50
	250	50	1045	678	0.65	13.56 ± 0.70*	25.5	6.5	17.0	76.5		82	13
	501**												0

*Significantly greater than the solvent control, $p<0.01$
**Toxic dose

TABLE 4-3. SISTER CHROMATID EXCHANGE IN CHINESE HAMSTER OVARY CELLS WITH METABOLIC ACTIVATION OF CPFB: TRIAL 2

TREATMENT	DOSE µg/mL (µL/mL)	TOTAL CELLS SCORED	# OF CHROMOSOMES	# OF SCE	SCE/ CHROMOSOME	SCE/CELL MEAN ± S.E.	TIME IN BrdU (hr)	CELL CYCLE STAGES (%) M1	M1+	M2	M2+	% SCE INCREASE OVER SOLVENT	CONFLUENCE % SOLVENT CONTROL
CONTROLS													
NEGATIVE:													
McCoys 5a		50	1046	415	0.40	8.30 ± 0.44	25.5	0.5	4.0	93.5	2.0		
SOLVENT: 10% PLURONIC®	(10)	50	1045	372	0.36	7.44 ± 0.40	25.5		3.0	93.5	3.5		100
POSITIVE: CP	1.50	20	418	1054	2.52	52.70 ± 2.98*	25.5		6.5	93.0	0.5	608	88
CPFB	100	50	1050	465	0.44	9.30 ± 0.41*	25.5	3.5	10.5	83.0	3.0	24	88
	150	50	1049	513	0.49	10.26 ± 0.54*	25.5	1.5	8.5	88.5	1.5	37	75
	200	50	1046	609	0.58	12.18 ± 0.58*	25.5	9.0	17.0	73.0	1.0	64	50
	250	50	1045	678	0.65	13.56 ± 0.70*	25.5	6.5	17.0	76.5		82	13
	501**												0

*Significantly greater than the solvent control, $p<0.01$
**Toxic dose

TABLE 4-5. CHROMOSOME ABERRATIONS IN CHINESE HAMSTER OVARY CELLS WITHOUT METABOLIC ACTIVATION OF CPFB

(Results from individual cultures)

			NUMBER AND TYPE OF ABERRATION[a]																
		CELLS SCORED	NOT COMPUTED			SIMPLE			COMPLEX							NO. OF ABERRATIONS PER CELL	% CELLS WITH ABERRATIONS	% CELLS WITH >1 ABERRATIONS
			TG	SG	UC	TB	SB	DM	ID	TR	QR	CR	D	R	CI			
CONTROLS																		
NEGATIVE:																		
McCoys 5a		100	6	1												0.00	0.0	0.0
SOLVENT:																		
10% pluronic® 10 µL/mL		100	8													0.00	0.0	0.0
POSITIVE:																		
MMC 1.00 µg/mL		25	12	2		4	4			3	4	1				0.64	40.0	12.0
CPFB																		
50.1 µg/mL	A	100	12	4												0.00	0.0	0.0
	B	100	5				2									0.02	2.0	0.0
100 µg/mL	A	100	6	2									1			0.01	1.0	0.0
	B	100	5	2		1										0.01	1.0	0.0
150 µg/mL	A	100	8	1		1	2									0.03	3.0	0.0
	B	100	8	2			1									0.01	1.0	0.0
200 µg/mL	A	100	14				3									0.03	2.0	1.0
	B	100	5		1											0.00	0.0	0.0

[a]See Appendix 4-A for definitions of chromosome aberrations

TABLE 4-6. CHROMOSOME ABERRATIONS IN CHINESE HAMSTER OVARY CELLS WITHOUT METABOLIC ACTIVATION OF CPFB

(Results pooled from replicate cultures)

	CELLS SCORED	NUMBER AND TYPE OF ABERRATION[a]													NO OF ABERRATIONS PER CELL	% CELLS WITH ABERRATIONS	% CELLS WITH >1 ABERRATIONS
		NOT COMPUTED			SIMPLE			COMPLEX									
		TG	SG	UC	TB	SB	DM	ID	TR	QR	CR	D	R	CI			
CONTROLS																	
NEGATIVE AND SOLVENT	200	10	5		1	2						1			0.02	2.0	0.0
POSITIVE: MMC 0.08 µg/mL	25	8			4	3			2	3	1				0.52	28.0*	20.0*
CPFB 200.0 µg/mL	200	19	7												0.00	0.0	0.0

[a] Significantly greater than the pooled negative and solvent controls, $p < 0.01$

[a] See Appendix 4-A for definitions of chromosome aberrations

TABLE 4-7. CHROMOSOME ABERRATIONS IN CHINESE HAMSTER OVARY CELLS WITHOUT METABOLIC ACTIVATION OF CPFB

(Results from individual cultures)

		CELLS SCORED	NUMBER AND TYPE OF ABERRATION[a]													NO. OF ABERRATIONS PER CELL	% CELLS WITH ABERRATIONS	% CELLS WITH >1 ABERRATIONS
			NOT COMPUTED			SIMPLE			COMPLEX									
			TG	SG	UC	TB	SB	DM	ID	TR	QR	CR	D	R	CI			
CONTROLS																		
NEGATIVE:																		
McCoys 5a		100	9	4		1	1									0.02	2.0	0.0
SOLVENT:																		
10% pluronic® 10 µL/mL		100	2	1			1						1			0.02	2.0	0.0
POSITIVE:																		
MMC 1 00 µg/mL		25	8			4	3			2	3	1				0.52	28.0	20.0
CPFB																		
200.0 µg/mL	A	100	14	4												0.00	0.0	0.0
	B	100	5	3												0.00	0.0	0.0

[a]See Appendix 4-A for definitions of chromosome aberrations

TABLE 4-8. CHROMOSOME ABERRATIONS IN CHINESE HAMSTER OVARY CELLS WITH METABOLIC ACTIVATION OF CPFB

(Results pooled from replicate cultures)

	CELLS SCORED	NUMBER AND TYPE OF ABERRATION*												NO. OF ABERRATIONS PER CELL	% CELLS WITH ABERRATIONS	% CELLS WITH >1 ABERRATIONS
		NOT COMPUTED			SIMPLE			COMPLEX								
		TG	SG	UC	TB	SB	DM	ID	TR	QR	CR	D	R	CI		
CONTROLS																
NEGATIVE AND SOLVENT	200	9	1												0.00	0 0	0 0
POSITIVE:																
MMC 25.0 µg/mL	25	2	3	2		2		1	2	1	1				0.28	20 0*	8 0*
CPFB																
100 µg/mL	200	6													0.00	0.0	0.0
150 µg/mL	200	4	4		1	1			1						0.02	1.5	0.0
200 µg/mL	200	10	3												0.00	0.0	0.0
250 µg/mL	200	9	5		3										0.02	1.0	0.5
300 µg/mL**																	

* Significantly greater than the pooled negative and solvent controls, $p < 0.01$.
** Toxic dose.
* See Appendix 4-A for definitions of chromosome aberrations.

TABLE 4-9. CHROMOSOME ABERRATIONS IN CHINESE HAMSTER OVARY CELLS WITH METABOLIC ACTIVATION OF CPFB

(Results from individual cultures)

		CELLS SCORED	NUMBER AND TYPE OF ABERRATION[a]													NO. OF ABERRATIONS PER CELL	% CELLS WITH ABERRATIONS	% CELLS WITH >1 ABERRATIONS
			NOT COMPUTED			SIMPLE			COMPLEX									
			TG	SG	UC	TB	SB	DM	ID	TR	QR	CR	D	R	CI			
CONTROLS																		
NEGATIVE:																		
McCoys 5a		100	6	1												0.00	0.0	0.0
SOLVENT:																		
10% pluronic® 10 µL/mL		100	3													0.00	0.0	0.0
POSITIVE:																		
MMC 1.00 µg/mL		25	2	3	2		2		1	2	1	1				0.28	20.0	8.0
CPFB																		
200 0 µg/mL	A	100	3													0.00	0.0	0.0
	B	100	3													0.00	0.0	0.0
150 µg/mL	A	100	1	1		1										0.01	1.0	0.0
	B	100	3	3			1			1						0.02	2.0	0.0
200 µg/mL	A	100	5	3												0.00	0.0	0.0
	B	100	5													0.00	0.0	0.0
250 µg/mL	A	100	4	4		3										0.03	2.0	1.0
	B	100	5	1												0.00	0.0	0.0
300 µg/mL*																		

[a]See Appendix 4-A for definitions of chromosome aberrations.

APPENDIX 4-A

DEFINITIONS OF CHROMOSOME ABERRATIONS FOR GIEMSA STAINED CELLS

NOT COMPUTED

TG Chromatid Gap:	("tid gap"). An achromatic (unstained) region in one chromatid, the size of which is equal to or smaller than the width of a chromatid. These are noted but not usually included in final totals of aberrations as they may not all be true breaks.
SG Chromosome Gap:	("isochromatid gap, IG"). Same as chromatid gap but at the same locus in both sister chromatids.
UC Uncoiled Chromosome:	Failure of chromatin packing. Probably not a true aberration.
PP Polyploid cell:	A cell containing multiple copies of the haploid number (n) of chromosomes. Only indexed if very common. Not counted in the cells scored for aberrations.
E Endoreduplication:	4n cell in which separation of chromosome pairs has failed. Only indexed if very common. Not counted in the cells scored for aberrations.

SIMPLE

TB Chromatid Break:	An achromatic region in one chromatid, larger than the width of a chromatid. The associated fragment may be partially or completely displaced.
SB Chromosome Break:	Chromosome has a clear break, forming an abnormal (deleted) chromosome with an acentric fragment that is dislocated. This classification now includes the acentric fragment (AF). The AF was different from the SB only in that it was not apparently related to any specific chromosome.
DM "Double Minute"	These are small double dots, some of fragment: which are terminal deletions and some interstitial deletions and probably small rings. Their origins are not distinguishable.

COMPLEX

ID Interstitial Deletion:	Length of chromatid "cut out" from midregion of a chromatid resulting in a small fragment or ring lying beside a shortened chromatid or a gap in the chromatid.
TR Triradial:	An exchange between two chromosomes, or one chromosome and an acentric fragment, which results in a three-armed configuration.
QR Quadriradial:	As triradial, but resulting in a four-armed configuration.

CR Complex Rearrangement:	An exchange among more than two chromosomes or fragments which is the result of several breaks.
D Dicentric:	An exchange between two chromosomes which results in a chromosome with two centromeres. This is often associated with an acentric fragment in which case it is classified as DF.
DF	Dicentric with fragment.
TC Tricentric:	An exchange involving three chromosomes and resulting in a chromosome with three centromeres. Often associated with two to three AF. Such exchanges can involve many chromosomes and are named as follows:
QC Quadricentric:	four centromeres, up to four AF
PC Pentacentric:	five centromeres, up to five AF
HC Hexacentric:	six centromeres, up to six AF
R Ring:	A chromosome which forms a circle containing a centromere. This is often associated with an acentric fragment in which case it is classed as RF.
RC Ring Chromatid:	Single chromatid ring (acentric).
RF	Ring with associated acentric fragment.
CI Chromosome	Exchange within a chromosome; e.g., a Intrachange:ring that does not include the entire chromosome.
T Translocation:	Obvious transfer of material between two chromosomes resulting in two abnormal chromosomes. When identifiable, scored as "T" not "2Ab."
AB	Abnormal monocentric chromosome. This is a chromosome whose morphology is abnormal for the karyotype, and often the result of a translocation, pericentric inversion, etc. Classification used if abnormality cannot be ascribed to; e.g., a reciprocal translocation.

OTHER

GT/>	A cell which contains more than 10 aberrations. A heavily damaged cell should be analyzed to identify the types of aberrations and may not actually have >10, e.g., multiple fragments such as those found associated with a tricentric.

SECTION 5

GENETIC TOXICOLOGY TEST ON CHLOROPENTAFLUOROBENZENE IN THE *IN VITRO* TRANSFORMATION OF BALB/C-3T3 CELLS ASSAY WITH AND WITHOUT METABOLIC ACTIVATION

Myhr, B.C.*

ABSTRACT

Chloropentafluorobenzene (CPFB) was assayed for its ability to induce morphological cell transformation in BALB/c-3T3 cell cultures in the absence and presence of a rat liver S9 metabolic activation system. Seven doses ranging from 50 to 600 µg/mL were examined as emulsions in Eagles Minimal Essential Medium (EMEM) containing 1% w/v Pluronic® F-68. The toxicity, determined from the clonal survival of ouabain-resistant cells in the presence of a wildtype monolayer, ranged from essentially 100% to 0% without S9 and 100% to 13.5% with S9. The number of transformed foci in the CPFB-treated cultures did not change from the corresponding negative controls. Therefore, CPFB was evaluated as negative for the induction of morphological transformation in BALB/c-3T3 cell cultures.

INTRODUCTION

BALB/c-3T3 mouse cells multiply in culture until a uniform monolayer is achieved and then cease further division (Kakunaga, 1973; Rundell, 1984). These nontransformed cells, if injected into immunosuppressed mice (1×10^7 cells/animal), do not produce neoplastic tumors (Kakunaga, 1973; Rundell et al., 1983; Rundell, 1984). However, BALB/c-3T3 cells treated *in vitro* with some chemical carcinogens give rise to foci of morphologically altered cells superimposed on the contact-inhibited cell monolayer. If foci picked from cell cultures are grown to larger cell numbers and are injected into immunosuppressed mice, a malignant tumor will be obtained in most cases. Thus, the appearance of foci of altered cells is correlated with malignant transformation.

The ability of BALB/c-3T3 cells to metabolize test articles from various chemical classes can be enhanced by the addition of an exogenous S9 metabolic activation system to the cultures during the treatment period. However, the standard treatment period of 72 h is reduced to only four hours because of S9 toxicity and the degradation of the NADPH-dependent P450 enzyme system, so this assay modification may not always detect procarcinogens. The procarcinogen dimethylnitrosamine (DMN) does not transform BALB/c-3T3 cells in the absence of S9 (Rundell et al., 1983), but DMN treatments with the S9 activation system usually induce statistically significant increases in the frequency of transformed foci (Matthews and Rundell). Similarly, S9-dependent induction of

transformed foci by the DMN has been reported for another mouse line (Tu et al., 1984).

An appropriate dose range for toxic test articles is selected by a novel method for determining clonal survival under the mass culture conditions of the transformation assay. Normal BALB/c-3T3 cells are sensitive to the cell membrane poison, ouabain, and are quickly killed. However, a mutant BALB/c-3T3 cell line has been established in this laboratory that is ouabain-resistant but otherwise as sensitive to test articles as the parent (wildtype), ouabain-sensitive cells. When a few (eg., 600) ouabain-resistant cells are mixed with a large number of wildtype cells (eg., 7×10^4 cells), the clonal survival of the ouabain-resistant cells can be determined by the addition of 4 mM ouabain to the culture medium after the treatment period. In this manner, the test article toxicity is determined under the same cellular exposure conditions that will occur in the transformation assay mass cultures.

The objective of this assay was to evaluate chloropentafluorobenzene (CPFB) for its ability to induce the malignant transformation of cultured BALB/c-3T3 mouse cells in the absence and presence of a rat liver S9 metabolic activation system. Transformation is recognized as a dense, piling up of morphologically altered cells, called a focus, superimposed on a monolayer of contact-inhibited cells (Heidelberger et al., 1983; Rundell, 1984; Rundell, 1984).

MATERIALS AND METHODS

Indicator Cells

Clone 1-13 of BALB/c-3T3 mouse cells was obtained from Dr. T. Kakunaga (Kakunaga, 1973). A subclone of these cells with a low spontaneous frequency of transformants was used for the transformation assays in this study. Stocks of cells were maintained in liquid nitrogen and have been checked to ensure the absence of mycoplasma contamination. Laboratory cell cultures were grown in EMEM supplemented with fetal bovine serum (FBS), L-glutamine, and antibiotics.

Controls

Negative (Solvent) control: The negative control (also referred to as the solvent control) was EMEM culture medium containing the emulsifier, Pluronic® F-68, at a concentration of 1% w/v. In addition, for the S9 activation assay, the S9 activation system was added to the medium.

Medium Control: A medium control consisting only of EMEM culture medium or medium containing the S9 activation system was also included with each assay. This control was used to detect any significant effects that might be caused by the 1% Pluronic® F-68 component.

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Positive Controls: A known carcinogen, 3-methylcholanthrene (MCA), was used at 2.5 µg/mL as a positive control for the transformation of BALB/c-3T3 cells in the absence of S9.

A second known carcinogen, dimethylnitrosamine DMN, was used as the positive control for the transformation of BALB/c-3T3 cells in the presence of the S9 activation system. DMN requires activation by S9 microsomal enzymes to become transforming, and two or more concentrations of DMN within the 1 to 10 µL/mL range were chosen to demonstrate a significant response for at least one concentration of DMN.

Test Material

The test material, CPFB, was a clear, colorless liquid. It was stored in a chemical cabinet at room temperature in its original glass container.

S9 Metabolic Activation System

S9: A 9000 x g supernatant fraction (Ames et al., 1984) prepared from the livers of Aroclor 1254-induced Sprague-Dawley male rats was purchased commercially and used for this study. The S9 was prescreened and selected for relatively low toxicity to BALB/c-3T3 cells and for the conversion of DMN to toxic substances. The concentration of S9 selected for the assay was 40 µL/mL, which corresponded to 1.4 mg S9 protein/mL in the treatment medium.

CORE: The S9 activation system included a NADPH regenerating system (CORE) composed of NADP and isocitric acid in the presence of S9. The final concentrations of the CORE components in the treatment medium were approximately 236 µg/mL of NADP (sodium salt) and approximately 1552 µg/mL of isocitrate. The CORE solution was prepared fresh as a 20X stock solution in culture medium and was then combined with S9 to give a 10X S9 mix. The mix was held on ice until used in the assay.

Experimental Design

Test Material Preparation: CPFB was found to be soluble in dimethylsulfoxide or tetrahydrofuran at approx. 100 mg/mL, but immiscible globules formed when the solutions were diluted 1:10 into culture medium (serum-free or with 10% fetal bovine serum). The globules settled to the bottom of the container and coalesced.

In order to achieve a more uniform presentation of CPFB to a cell monolayer bathed in culture medium, Pluronic® F-68 was investigated as an emulsifying agent. This polyalcohol is frequently used in cell cultures due to its low toxicity and ability to lower surface tension. When a preparation of 10 mg/mL of CPFB in 9% w/v Pluronic® F-68 in deionized water was vigorously agitated with a Tissumizer®, a stable, milky white emulsion was formed. The CPFB remained dispersed into tiny droplets after diluting 1:10 into culture medium and did not coalesce, even though the dense

droplets settled and collected on the bottom of the container. A brief mixing by vortexer caused complete dispersion.

It was also noted that CPFB scores and softens a polystyrene surface, whether placed directly on the surface or as an emulsion with Pluronic® F-68. Thus, emulsified CPFB appeared to be available to interact with the cells, and only glass containers, pipets and culture bottles were used for this study.

For both the dose rangefinding study and the transformation assays, CPFB was prepared fresh as a 50 mg/mL emulsion in 10% w/v Pluronic® F-68. The Tissumizer® was operated for approximately 1 min at a setting of 40-50. Lower concentrations were prepared by dilutions into 10% w/v Pluronic® F-68. The treatment media were prepared by diluting the Pluronic® F-68 emulsions 1:10 into EMEM culture medium so that the Pluronic® F-68 was diluted to 1% w/v.

Preliminary Dose Rangefinding: Glass culture bottles with approximately 60 cm^2 of surface area were seeded concurrently with approximately 600 ouabain-resistant 3T3 cells and 7×10^4 wildtype cells. The following day, one culture was exposed to each of nine doses with and without S9, starting at 5000 µg/mL and diluting in 2-fold steps. Two solvent control cultures containing 1% w/v Pluronic® F-68 in EMEM culture medium were prepared for both test conditions. After an exposure period of approx. four hours in the presence of the S9 activation system or approximately 72 h without S9, the cells were washed with a physiological solution and refed with EMEM culture medium containing 4 mM ouabain. The cultures were refed with medium containing 4 mM ouabain four to five days later. Surviving colonies were terminated 7-10 days after the treatment periods, stained with Giemsa, and counted manually.

A relative survival for each treatment condition was obtained by comparing the number of surviving colonies to the average colony count for the solvent control cultures. This survival information was used to select seven doses for the transformation assay that would span an anticipated survival range of 10% to 100%.

Transformation Assay: The transformation assay procedure was adapted from that reported by Kakunaga (Kakunaga, 1973). Glass culture bottles having a monolayer growth area of approximately 60 cm^2 were used. Each bottle was seeded with approximately 7×10^4 cells for the transformation assay and 7×10^4 cells plus approximately 600 ouabain-resistant cells for the concurrent clonal survival assay. On the day after seeding, nine cultures were exposed to each selected treatment with CPFB and the positive controls. Eighteen cultures were used as solvent controls for each of the test conditions (with and without the S9 activation system). In addition, nine cultures were used as EMEM medium controls without Pluronic® F-68 for both test conditions. One mixed culture of wildtype and ouabain-resistant cells was exposed to each treatment and control condition.

The treatments were conducted at 37 ± 1°C for approximately four hours with the S9 activation system and approximately 72 h without S9. The treatments were initiated by adding 1.0 mL of a 10X suspension of CPFB or solution of positive control to each culture containing 9 mL of culture medium (with or without the S9 activation system). After treatment, all cultures were washed with Hanks' balanced salt solution. The transformation assay cultures were refed with fresh EMEM culture medium, and the incubation was continued for approximately four weeks with refeeding twice a week. The clonal survival cultures were refed with medium containing 4 mM ouabain. The cultures without S9 were refed with 4 mM ouabain four days later and were terminated seven days after treatment. The S9 activation cultures were simply terminated seven days after treatment.

The cultures were terminated by fixing the cells with methanol and staining with 10% Giemsa in tap water. Stained cultures were examined by eye and by microscope to determine the number of foci of transformed cells and the colony survival. The transformation assay cultures were coded with random numbers prior to evaluation for foci.

Evaluation of Transformed Foci: At the end of the incubation period, cultures of cells with a normal phenotype yield a uniformly stained monolayer of round, contact-inhibited cells. Transformed cells form a multi-layered mass of cells, or focus, that stains deeply and is superimposed on the surrounding monolayer of cells (Kakunaga, 1973, Rundell, 1984). The foci are variable in size and exhibit several variations in morphological features. Many scored foci consist of a dense piling-up of cells with a random, criss-cross orientation of fibroblastic cells at the periphery of the focus and extensive invasiveness into the contiguous monolayer. Other scored foci are composed of more rounded cells with little criss-crossing at the periphery but with necrosis at the center caused by the dense piling-up of a large number of cells. A third variation is a focus without the necrotic center and large number of cells but which exhibits the criss-cross pattern of overlapping cells throughout most of the colony.

Some deeply stained areas are not scored as transformed foci because the random orientation of fibroblastic cells is not observed. Microscopic examination is routinely employed for scoring and for the final judgement of the transformed character of each focus.

All foci that exhibited the transformed characteristics were scored. In the raw data, a record of focus size was maintained by scoring foci greater than four mm in diameter as + + + and those of two to four mm in diameter as + +. No significance is currently attached to this categorization. The sum of all scored foci (+ + + and + +) was reported for each culture and was used for the assay analysis.

Assay Acceptance Criteria

The clonal survival assay conducted simultaneously with the transformation assay was considered acceptable for evaluation of the test results by meeting the following three criteria:

1. The negative (solvent) control cultures must have macroscopically visible BALB/c-3T3 cell colonies representing a cloning efficiency of 15% or greater.
2. At least one of the test material treatments resulted in ≥ 50% cell survival.
3. A cytotoxic dose response was obtained for the test material treatments, unless the test material was nontoxic at 10 mg/mL or its solubility limit in culture medium was exceeded.

The transformation assay was considered acceptable for evaluation of test results by meeting the following five criteria:

1. Negative (solvent) control, positive control, and test material treatments resulted in contiguous monolayers of cells to be evaluated.
2. Negative control spontaneous frequencies of transformation did not exceed an average of approximately two foci per culture.
3. At least one of the positive control treatments resulted in an average number of foci per culture vessel that was significantly different from the negative control at the 99% confidence level ($p \leq 01$)
4. A minimum number of six culture vessels per test condition was available for analysis.
5. A minimum number of three treatment levels of the test material was available for analysis.

In addition, the cytotoxicity dose-related data from the preliminary and simultaneous clonal survival assays should be qualitatively similar over a comparable range of test chemical treatments.

Assay Evaluation Criteria

The appearance of transformed foci usually occurs as a general increase in foci for all cultures exposed to a transforming dose. However, large numbers of foci may appear at random in one or more culture vessels in a treatment set, resulting in skewing of the mean number of foci in that set. This skewing could be caused by factors such as mechanical disruption and respreading of transformed foci cells or a culture-conditioning effect caused by the early appearance of a focus. The appearance of occasional dishes with numerous foci is a random process and occurs in both treated and control cell cultures. In our laboratory, we have utilized a $\log_{10}$ mathematical transformation to handle this non-normal distribution of BALB/c-3T3 cell transformed foci data (Rundell et al., 1983); however, other mathematical models have also been proposed (Whorton et al., 1982). After performing a $\log_{10}$ transformation of the data, Bailey's modification of the Student's t-test (Bailey et al., 1959) was used to evaluate positive control and test chemical treatment transforming activity for significant differences from the negative control. The possible spectrum of responses was routinely subdivided into three levels for the evaluation of each treatment.

EVALUATION OF INDIVIDUAL TREATMENTS

Strong positive response	=	$p \leq .01$
Weak positive response	=	$p \leq .05$
Negative response	=	$p > .05$

The results of each treatment condition were evaluated in relation to the observed activities of model compounds, and scientific judgement was exercised in the evaluation of each test material. In general, a response at only one dose attaining a 95% confidence level is not considered sufficient evidence for activity in this assay. However, responses at two or more treatment levels attaining the 95% confidence level and exhibiting evidence of a dose relationship are considered as evidence for transformation. Responses achieving the 99% confidence level for one or more test material treatments are usually considered sufficient for a positive evaluation.

RESULTS AND DISCUSSION

Clonal Survival

As shown in Table 5-1, CPFB was highly toxic or lethal to BALB/c-3T3 cells at 625 µg/mL and higher concentrations. Little or no toxicity was obtained at 156 µg/mL. These results were essentially unchanged by the addition of the rat liver S9 metabolic activation system (Table 5-2). Thus, a dose range of 100 to 600 µg/mL covered the entire survival range appropriate for the transformation assay with or without S9. Six doses were chosen at 100 µg/mL increments over this range, and a seventh dose (50 µg/mL) was also included to help define the variation in response for nontoxic treatments.

Transformation Assay Without S9

Table 5-3 summarizes the results obtained for CPFB in the transformation assay without S9. For treatments with 50 to 500 µg/mL (approximately 100% survival to 2% survival), the frequency of transformed foci remained equivalent to the solvent control. At 600 µg/mL, the clonal survival was zero but the transformation assay cultures recovered over the four-week culture period. An apparent increase in transformed foci was caused by one culture (colony splitting was likely) but the total number of foci still remained below the medium negative control level. In contrast, the MCA positive control induced a significant increase in focus formation. Thus, no evidence was obtained for transforming activity by CPFB without S9 in BALB/c-3T3 cell cultures.

Transformation Assay with S9

Table 5-4 summarizes the results obtained for CPFB in the transformation assay with S9. The frequency of transformed foci in the cultures exposed to CPFB remained strictly comparable to the solvent or medium negative controls. An apparent increase for the nontoxic treatment with

200 µg/mL was due to one culture. No increases were observed for the toxic range of treatments (400, 500 and 600 µg/mL). Both of the DMN positive controls clearly induced foci. The addition of S9 activation conditions therefore did not confer any transforming activity to CPFB.

CONCLUSION

The test material, CPFB, was evaluated using seven doses from 50 to 600 µg/mL, both in the absence and presence of a rat liver S9 metabolic activation system. A wide range of toxic response was obtained, but no significant changes in the frequency of transformed foci were observed. Therefore, under the conditions of this study, CPFB was evaluated as negative for transforming BALB/c-3T3 cells in culture.

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TABLE 5-1. CYTOTOXIC ACTIVITY OF CHLOROPENTAFLUOROBENZENE IN THE PRELIMINARY CLONAL SURVIVAL ASSAY WITHOUT S9 ACTIVATION

TREATMENT CONDITION		COLONIES/ CULTURE	RELATIVE CELL SURVIVAL %
Solvent Control[a]		211,286	
	Average	248.5	100.0
CPFB:			
19.5 µg/mL		248	99.8
39.1 µg/mL		286	115.1
78.1 µg/mL		301	121.1
156 µg/mL		239	96.2
313 µg/mL		173	69.6
625 µg/mL		10	4.0
1250 µg/mL		0	0
2500 µg/mL		0	0
5000 µg/mL		0	0

[a]EMEM culture medium containing 1% Pluronic® F-68

TABLE 5-2. CYTOTOXIC ACTIVITY OF CHLOROPENTAFLUOROBENZENE IN THE PRELIMINARY CLONAL SURVIVAL ASSAY WITH S9 ACTIVATION

TREATMENT CONDITION		COLONIES/ CULTURE	RELATIVE CELL SURVIVAL %
Solvent Control[a]		254,265	
	Average	259.5	100.0
CPFB:			
19.5 µg/mL		206	79.4
39.1 µg/mL		282	108.7
78.1 µg/mL		218	84.0
156 µg/mL		234	90.2
313 µg/mL		96	37.0
625 µg/mL		0	0
1250 µg/mL		0	0
2500 µg/mL		0	0
5000 µg/mL		0	0

[a]EMEM culture medium containing 1% Pluronic® F-68

TABLE 5-3. TRANSFORMING ACTIVITY OF CHLOROPENTAFLUOROBENZENE ASSESSED IN THE TRANSFORMATION ASSAY USING BALB/c-3T3 CELLS WITHOUT S9 ACTIVATION

TREATMENT CONDITION	COLONY COUNT[a]	RELATIVE SURVIVAL[b] (%)	FOCUS DATA			TRANSFORMING[c] ACTIVITY
			TOTAL CULTURES	TOTAL FOCI	AVERAGE FOCI/ CULTURE	MEAN FOCI/ CULTURE
Solvent Control[d]	183	100.0	18	11	0.61	0.27
Positive Control[e]	72	35.8	9	151	16.8	15.6**
Medium Control[f]	201	109.8	9	27	3.0	0.82
CPFB:						
50 µg/mL	175	95.6	9	4	0.44	0.32
100 µg/mL	186	101.6	9	3	0.33	0.26
200 µg/mL	173	94.5	9	3	0.33	0.26
300 µg/mL	121	66.1	9	2	0.22	0.17
400 µg/mL	53	29.0	9	3	0.33	0.26
500 µg/mL	4	2.2	9	2	0.22	0.17
600 µg/mL	0	0	9	21	2.33	0.81

[a] Clonal survival assay performed concurrently with the transformation assay
[b] Colony survival relative to the solvent control, except for the positive control, which is relative to the medium control
[c] The mean transforming activity is expressed as the anti-log of the $\log_{10}$ mean minus one
[d] Solvent Control: EMEM culture medium containing 1% Pluronic® F-68
[e] Positive Control: The positive control treatment was 3-methylcholanthrene at 2.5 µg/mL
[f] Medium Control: EMEM culture medium without Pluronic® F-68
**$p \leq 0.01$

TABLE 5-4. TRANSFORMING ACTIVITY OF CHLOROPENTAFLUOROBENZENE ASSESSED IN THE TRANSFORMATION ASSAY USING BALB/c-3T3 CELLS WITH S9 ACTIVATION

TREATMENT CONDITION	COLONY COUNT[a]	RELATIVE SURVIVAL[b] (%)	FOCUS DATA			TRANSFORMING[c] ACTIVITY
			TOTAL CULTURES	TOTAL FOCI	AVERAGE FOCI/ CULTURE	MEAN FOCI/ CULTURE
Solvent Control[d]	148	100.0	18	27	1.50	0.50
Positive Control[e]						
DMN, 2 µL/mL	40	25.6	8	43	5.38	3.63**
DMN, 4 µL/mL	12	7.7	9	28	3.11	2.49**
Medium Control[f]	156	105.4	9	4	0.44	0.32
CPFB:						
50 µg/mL	149	100.7	9	10	1.11	0.88
100 µg/mL	152	102.7	9	6	0.67	0.59
200 µg/mL	150	101.4	9	16	1.78	0.88
300 µg/mL	165	111.5	9	3	0.33	0.26
400 µg/mL	114	77.0	9	6	0.67	0.59
500 µg/mL	73	49.3	9	7	0.78	0.56
600 µg/mL	20	13.5	9	2	0.22	0.17

[a] Clonal survival assay performed concurrently with the transformation assay
[b] Colony survival relative to the solvent control, except for the positive control, which is relative to the medium control
[c] The mean transforming activity is expressed as the anti-log of the $\log_{10}$ mean minus one
[d] Solvent Control: EMEM culture medium containing 1% Pluronic® F-68
[e] Positive Control: The positive control treatment was 3-methylcholanthrene at 2.5 µg/mL
[f] Medium Control: EMEM culture medium without Pluronic® F-68
**$p \leq 0.01$

SECTION 6

MUTAGENICITY TEST ON CHLOROPENTAFLUOROBENZENE IN THE *IN VIVO/IN VITRO* RAT PRIMARY HEPATOCYTE UNSCHEDULED DNA SYNTHESIS ASSAY

Cifone, M.A.[a]

ABSTRACT

In the *In Vivo/In Vitro* Rat Primary Hepatocyte Unscheduled DNA Synthesis Assay, the test material, chloropentafluorobenzene (CPFB), did not induce significant changes in the nuclear labeling of rat hepatocytes over a dose range of approximately 5000 to 625 mg/kg. Three male Fischer 344 (F-344) rats were treated by oral gavage at each of four doses with the test material solubilized in corn oil. At 4.0 to 4.9 h after treatment with the test material, primary hepatocyte cultures were prepared. Viabilities of the hepatocytes ranged from 75.5% to 96.3%. After attachment, the cultures were incubated with 10 µCi/mL ^{3}HTdr for four hours. The cultures were prepared for analysis of nuclear labeling about 17 h after removal of the radioactivity and addition of 0.17 mM thymidine. None of the criteria used to indicate UDS was approached by the treatments and no dose-related response was observed. The test material, CPFB, was therefore evaluated as inactive in the *In Vivo/In Vitro* Rat Primary Hepatocyte Unscheduled DNA Synthesis Assay.

INTRODUCTION

This assay is designed to measure unscheduled DNA synthesis (UDS) in rat liver cells (hepatocytes) using the autoradiographic technique described by Williams, (1980). Hepatocytes were isolated from the livers of rats exposed *in vivo* to the test article. Only a small percentage of the cells enter S-phase (replicative DNA synthesis) during the exposure period, so the incorporation of ^{3}HTdr into DNA during *in vitro* culturing, as analyzed by autoradiography, may be used as a measure of the repair of DNA damage caused by treatment with the test article. This UDS measurement of DNA repair appears to correlate well with known mutagenic or carcinogenic activities of chemicals (Williams, 1977).

The objective of this assay was to detect DNA damage caused by the test material, or an active metabolite, by measuring UDS induced in rat primary hepatocytes *in vivo*. The existence and degree of DNA damage was inferred from an increase in net nuclear grain counts in hepatocytes obtained from treated animals when compared to those from untreated animals. The types of DNA damage are unspecified but must be recognizable by the cellular repair system and result in the incorporation of new bases (including ^{3}H-thymidine) into DNA.

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METHODS AND MATERIALS

Indicator Cells

The indicator cells for this assay were hepatocytes obtained from adult male F-344 rats (CDF), weighing 189.8 to 208.3 g, purchased from Charles River Breeding Laboratories, Incorporated. The animals scheduled for this assay were fed Purina Certified Rodent Chow (Formula 5002) and water ad libitum. Animals were quarantined a minimum of five days prior to random assignment to the study and identification by ear tag.

Animals were anesthetized prior to surgery using about 60 mg sodium pentobarbitol (V-Pento)/kg and were exsanguinated during the liver perfusion.

The cells were obtained by perfusion of the livers *in situ* with a collagenase solution. Monolayer cultures were established in culture dishes and were used the same day for analysis of the UDS activity. All cultures were maintained as monolayers at about 37°C in a humidified atmosphere containing approximately 5% CO_2.

Media

The cell cultures were established in Williams' Medium E supplemented with 10% fetal bovine serum, 2mM L-glutamine, 100 µg/mL streptomycin sulfate, and 150 µg/mL gentamicin (WME+). WME+ without serum is referred to as WMEI. After the establishment period, cultures were refed with WMEI containing 10 µCi/mL ^{3}HTdr, 40-60 Ci/mMole (WME-treat).

Vehicle control

The test article was suspended in Mazola 100% pure corn oil (Lot 021489). A vehicle negative control consisting of three rats treated by oral gavage with corn oil was performed. Dosing volumes did not exceed 5.0 mL/kg.

Positive control

The positive control compound is known to induce UDS *in vivo* in rat hepatocytes. Dimethylnitrosamine (DMN, CAS 62-75-9) at approximately 10 mg/kg was used. Three rats were treated by intraperitoneal injection.

Test Material

The test material, CPFB, was a clear colorless liquid. It was stored in a chemical cabinet at room temperature in its original glass container.

Dosing Procedure

For the UDS assay, rats were treated by oral gavage with the test article suspended in corn oil. The total volume of the test article solution administered did not exceed 5.0 mL/kg. Fresh preparations of test article in vehicle were used for any testing purpose.

Dose Selection and Perfusion Time

For the UDS assay, the highest dose selected was 5000 mg/kg. Three additional doses of test material were chosen using approximate two-fold dilution steps and a minimum of three animals per dose. One timepoint for UDS was performed at 4.0 to 4.9 h after the administration of a single dose of the test article by oral gavage. The timepoint stated in the protocol is approximately 4.0 h but some of the animals arrived at the facility late and the hepatocytes were collected up to 4.9 h after dosing (doses 5000, 2500 and 1250 mg/kg). The difference in time was slight and did not affect the results.

UDS Assay

This assay was based on the procedures in rats described by Williams (1980), Mirsalis, Tyson and Butterworth (1982) and Butterworth et al. (1987). The hepatocytes were obtained by perfusion of livers *in situ* for about four minutes with Hanks' balanced salts (Ca^{++} - Mg^{++}-free) containing 0.5 mM ethyleneglycol-bis (-aminoethyl ether)-N, N-tetraacetic acid (EGTA), and HEPES buffer at pH 7.2. Then WMEI containing 50-100 units/mL of collagenase was perfused through the liver for 11 min. The hepatocytes were obtained by mechanical dispersion of excised liver tissue in a culture dish containing the WMEI culture medium and collagenase. The suspended tissue and cells were allowed to settle to remove cell clumps and debris. The cell suspension was centrifuged and the cell pellet resuspended in WME +. After obtaining a viable cell count, a series of 35-mm culture dishes (at least two per animal containing a 25-mm round, plastic coverslip and at least one per animal to assess attachment efficiency) was inoculated for each animal with approximately 0.5×10^6 viable cells in 3 mL of WME + per dish. Routinely, at least nine plates are prepared from each animal, but in some cases low recovery results in fewer plates being prepared. This does not affect the assay as long as sufficient cells are available for analysis.

An attachment period of 1.5 to 2.0 h at 37°C in a humidified atmosphere containing 5% CO_2 was used to establish the cell cultures. Unattached cells were then removed and the cultures were refed with 2.5 mL WME-treat. Three of the replicate cultures from each animal were used for the UDS assay; two of the replicates were used to assess attachment unless only one plate was available due to low cell recovery or attachment. Any remaining cultures were kept for analysis in the event of technical problems with autoradiography.

Attachment efficiency was determined for at least one culture from each animal using trypan blue dye exclusion and *in situ* analysis.

After a labeling period of four hours, labeled cell cultures were refed with WMEI containing 0.17 mM thymidine and returned to the incubator for 16.7 to 17.1 h. The thymidine concentration was slightly lower than stated in the protocol due to technical error but the difference was small and did not affect the assay. Nuclei were then swollen by addition of 1% sodium citrate to the coverslips (containing the cell monolayers) for 10 min. The cells were next fixed in three changes of acetic acid:ethanol (1:3) and dried for at least 24 h. The fixed coverslips were mounted on glass slides, dipped in Kodak NTB2 emulsion, and dried. The emulsion coated slides were stored for seven days at 4°C in light-tight boxes containing Drierite. The emulsions were then developed in D19, fixed, and stained with Williams' modified hematoxylin and eosin procedure.

The cells were examined microscopically at approximately 1500 x magnification under oil immersion and the field was displayed on the video screen of an automatic counter. UDS was measured by counting nuclear grains and subtracting the average number of grains in three nuclear-sized areas adjacent to each nucleus (background count). This value is referred to as the net nuclear grain count. The coverslips were coded to prevent bias in grain counting.

The net nuclear grain count was determined for fifty randomly selected cells on each coverslip (three coverslips per animal) unless otherwise indicated. Only nuclei with normal morphologies were scored, and any occasional nuclei blackened by grains too numerous to count were excluded as cells in which replicative DNA synthesis occurred rather than repair synthesis. The mean net nuclear grain count was determined from the triplicate coverslips (150 total nuclei) for each animal and each treatment condition.

Occasionally, a coverslip is recounted at a later date by a different technician. Since a different cell population will generally be scored, the average count for fifty cells will be used in the calculation of the mean for the triplicate treatment.

Assay Acceptance Criteria

An assay normally will be considered acceptable for evaluation of the test results only if all of the criteria listed below are satisfied. This listing may not encompass all test situations, so the study director must exercise scientific judgment in modifying the criteria or considering other causes that might affect assay reliability and acceptance.

1. The viability of the hepatocytes collected from the perfusion process normally exceeds 70%. A variety of factors can affect cell yield and viability, so values below 70% are not uncommon nor necessarily detrimental. Toxicity of treatment with test article may be reflected in perfusion viability, therefore no lower limit will be set.

2. The viability of the monolayer cell cultures used for the assay treatments must be 70% or greater. Normally, the viability of attached cells is about 85%.

3. The positive control is used to demonstrate that the cell population employed was responsive and the methodology was adequate for the detection of UDS. For test materials causing weak or no UDS activity, the average response to the positive control treatments must exceed both criteria used to indicate UDS. For test materials clearly causing a dose-related UDS activity, an assay will be acceptable in the absence of a positive control lost for technical reasons. Historical control values for the positive control are NNG = 26.65 ± 7.69 (range, 15.47 to 37.25) and % ≥ 5 grains per nucleus = 93.1% ± 6.2% (range, 83.3% to 100.0%). Historical control values for the negative control are NNG = -0.81 ± 1.18 (range, -3.27 to 0.47) and % ≥ 5 grains per nucleus = 0.23% ± 0.35% (range, 0% to 0.7%).

4. Grain count data obtained per animal is acceptable as part of the evaluation if obtained from two replicate cultures and at least 50 nuclei per culture. Grain count data should be available from two of the three animals treated.

5. A minimum of three doses will be analyzed for nuclear grain counts. Repeat trials need only augment the number of analyzed doses in the first trial to achieve a total of three concentrations, but must include at least one dose previously assayed as acceptable.

Assay Evaluation Criteria

Several criteria have been established which, if met, provide a basis for evaluation of a test material as active in the UDS assay. The criteria for a positive response are based on a statistical analysis of the historical data as described by Casciano and Gaylor (1983).

The test material is considered active in the UDS assay at doses that cause:

1. An increase in the mean net nuclear grain count to at least five grains per nucleus above the concurrent negative control value, leading to a positive number and/or

2. An increase in the percent of nuclei having five or more net grains such that the percentage of these nuclei in test cultures is 10% above the percentage observed in the negative control cultures

Generally, if the first condition is satisfied, the second will also be met. However, satisfaction of only one condition can also indicate UDS activity. Different DNA-damaging agents can give a variety of nuclear labeling patterns, and weak agents may strongly affect only a minority of the cells. Therefore, both of the above conditions are considered in an evaluation. If the vehicle control shows an average less than -5.00 or more than 1.00 grains per nucleus, the assay will normally be considered invalid.

The test material is considered inactive in this assay if:

a. The mean net nuclear grain counts for all dosed groups is less than 1.0 net nuclear grain count above the concurrent negative control value and/or

b. The percent of nuclei with five or more net grains does not increase more than 2% above the concurrent negative control.

When results are neither clearly positive nor clearly negative, the presence of a dose response, the frequency distribution of cellular responses, and the reproducibility of data among slides is considered; the test article is then classified as "negative", "weak positive" or "equivocal". Groups in which one out of three animals show increases will be decided on a case by case basis depending on the level of activity in cells from the active animal, the level of activity in cells from the inactive animals and the presence or absence of activity in surrounding groups.

The positive control nuclear labeling is not used as a reference point to estimate mutagenic or carcinogenic risk associated with the UDS activity of the test material. UDS elicited by test agents in this assay is probably more dependent on the type of DNA damage inflicted and the available repair mechanisms than on the potency of the test agent as a mutagen or carcinogen. Some forms of DNA damage are repaired without the incorporation of new nucleic acids. Thus, the positive controls are used to demonstrate that the cell population employed was responsive and the methodology was adequate for the detection of UDS.

RESULTS AND DISCUSSION

The test article, CPFB, 99 + %, was solubilized in corn oil at concentrations ranging from 1004 to 125.3 mg/mL. All dosing stocks were prepared just prior to use. Each dose was prepared by dilutions of a primary stock with the vehicle. The test material appeared to be soluble in the vehicle at all concentrations prepared for dosing. Three rats per dose were treated with approximately 5000, 2500, 1250 and 625 mg/kg in volumes which did not exceed 5.0 mL/kg.

Perfusions were initiated 4.0 to 4.9 h after administration of a single dose of the test article by oral gavage. The hepatocytes collected for the UDS assay ranged in viability (determined by trypan blue exclusion) from 75.5% to 96.3% of the total cells collected in the perfusate (Table 6-1). Cells from one of the animals dosed with 5000 mg/kg of the test material had a low attachment efficiency and could not be analyzed. The attachment efficiencies of the remaining cultures varied from 22.4% to 90.3% (apparently unrelated to treatment toxicity) and the viability of the attached cells was very good, ranging from 81.3% to 97.4%.

The minimum criteria for a UDS response in this assay were determined by comparison to the averages of the concurrent vehicle control treatments. A positive response consisted of mean net nuclear grain counts exceeding 4.78, or at least 13.6% of the nuclei containing five or more grains. None of the treatments with the test material samples caused nuclear labeling significantly different from the vehicle control (see Table 6-2). Furthermore, no dose-related trend was evident. In contrast, the DMN treatments induced large increases in nuclear labeling that greatly exceeded both criteria

used to indicate UDS. Since the positive control animals were responsive to DMN, the test results were considered to provide conclusive evidence for the lack of UDS induction by the test material samples.

Heavily-labeled nuclei (blackened with numerous grains) represent cells undergoing DNA replication as opposed to DNA repair. The number present in this study was low and did not interfere with the assay. Only 168 cells (0.7%) among the 24,000 cells screened in the entire assay were heavily labeled.

CONCLUSION

The test material, CPFB, 99 + % did not induce significant changes in the nuclear labeling of rat primary hepatocytes for a dose range of about 625 to 5000 mg/kg. CPFB was therefore evaluated as inactive in the *In vivo/In Vitro* Rat Primary Hepatocyte UDS Assay

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TABLE 6-1. CULTURE DATA SUMMARY FOR HEPATOCYTES FROM RATS TREATED WITH CHLOROPENTAFLUOROBENZENE

Target Dose (mg/kg)	Animal Number	Perfusion[a] Viability (%)	Attachment[b] Efficiency (%)	(%) Viability[b]
VC	3251	86 4	74 8	90 0
VC	3255	92 0	66 7	97 4
VC	3264	92 0	77 4	90 3
625	3224	94 8	27 9	81 7
625	3256	90 9	22 4	81 3
625	3265	91 5	69 5	87 2
1250	3254	89 1	69 1	88 7
1250	3257	76 2	58 1	81 8
1250	3259	96 3	73 6	89 4
2500	3223	93 8	74 4	93 3
2500	3253	95 9	88 4	93 4
2500	3260	87 8	90 3	94 1
5000	3225	85 4	73 0	89 9
5000	3261	75 5	9 3	96 0
5000	3263	89 3	37 1	86 0
DMN	3252	90 6	71 4	87 1
DMN	3258	78 9	58 7	82 8
DMN	3262	90 5	75 0	96 0

[a] At time of hepatocyte collection (determined by trypan blue exclusion)

[b] Two culture dishes per animal were counted *in situ* after the attachment period using dilute trypan blue to determine viability; viabilities from animal #s 3261, 3257, 3224 and 3258 were determined from one culture dish

DMN = Positive control dimethylnitrosamine, 10 mg/kg

VC = Vehicle control 5 mL/kg of corn oil

TABLE 6-2. UDS DATA SUMMARY FOR HEPATOCYTES FROM RATS TREATED WITH CHLOROPENTAFLUOROBENZENE

Target Dose (mg/kg)	Animal Number	Mean (± S.D.) Net Nuclear Grains[a]	Mean Cyto Grains[b]	% Nuclei with ≥ 5 Net Grains	% S[c]
VC	3251	-0.01 (±0.89)	7.55	6.7	0.13
VC	3255	-0.38 (±0.30)	8.55	1.3	0.53
VC	3264	-0.27 (±0.22)	6.58	2.7	0.40
625	3224[d]	-0.49 (±0.64)	6.77	0.0	0.80
625	3256[d]	0.05 (±0.81)	4.83	0.0	0.50
625	3265	-0.18 (±0.64)	6.75	2.7	0.47
1250	3254	0.00 (±0.36)	5.81	2.7	0.40
1250	3257[d]	0.05 (±0.44)	6.94	4.0	0.70
1250	3259	-0.22 (±0.20)	6.51	2.0	0.66
2500	3223	-0.62 (±0.76)	5.77	0.7	0.73
2500	3253	-0.97 (±0.60)	7.95	0.7	0.80
2500	3260	-1.51 (±0.63	8.13	1.3	0.40
5000	3225	-0.32 (±0.53)	6.77	3.0	0.80
5000	3261	not analyzed due to poor attachment			
5000	3263	-0.30 (±1.00)	7.02	3.3	3.60
DMN	3252	19.44 (±0.75)	8.43	100.0	0.40
DMN	3258	24.03 (±4.22)	4.99	98.7	0.47
DMN	3262[e]	29.75 (±7.98)	5.26	96.0	0.07

[a] UDS = Mean net nuclear grain count from triplicate coverslips (150 total cells)
[b] Triplicate coverslips (150 total cells)
[c] Percent of S phase nuclei on triplicate coverslips (1500 total cells scored)
[d] UDS = Mean net nuclear grain count from duplicate coverslips (100 total cells for UDS and cytoplasmic grains, 1000 cells for S phase)
[e] UDS = Mean net nuclear grain count from duplicate coverslips (150 total cells for UDS and cytoplasmic grains, 1500 cells for S phase)
VC = Vehicle Control, 5 mL/kg of corn oil
DMN = Positive control dimethylnitrosamine, 10 mg/kg

SECTION 7

CHLOROPENTAFLUOROBENZENE GENOTOXICITY SUMMARY EVALUATION

Myhr, B.C.[a]

Chloropentafluorobenzene (CPFB), a fully substituted halogenated benzene, was tested for potential genotoxic activity by application to several *in vitro* assays and one *in vivo* assay for DNA damage. Because the substance has little or no solubility in aqueous medium, the polyalcohol Pluronic® F68 was used as an emulsifying agent for the *in vitro* assays. After homogenization 10% w/v Pluronic® F68, a stable suspension of tiny droplets was obtained that did not coalesce when diluted into culture media at Pluronic® F68 concentrations of 0.8-1% w/v. Glass containers and pipets were used for CPFB emulsions and treated cultures because the CPFB emulsions were found to score and soften polystyrene surfaces. For the *in vivo* study, CPFB was solubilized in corn oil and administered by oral gavage.

The Ames *Salmonella* reverse mutation assay was performed with the preincubation methodology in order to maximize contact between the bacterial and CPFB emulsions. Strains TA98, TA100, TA1535, TA1537 and TA1538 were used in the presence and absence of a rat liver S9 metabolic activation system (Aroclor 1254-induced). Doses up to 10,000 µg/plate were tested with S9, but lethality limited the maximum dose to 5000 µg/plate without S9. Toxicity was clearly evident for all test conditions at 1000 µg/plate. CPFB caused no increases in revertants and thus showed no mutagenic activity.

In mammalian cell culture, CPFB was tested for mutagenic activity at the HGPRT locus in CHO cells. After a four-hour exposure in the presence and absence of the rat liver S9 activation system, excessive toxicity was obtained at concentrations above 500 µg/mL. Without S9, a CPFB concentration range of 10 µg/mL to 500 µg/mL yielded a survival range of 93% to 7.5% but no significant increases in mutant frequency. Two trials were performed with S9 activation in order to interpret assay fluctuations after toxic treatments. Over an inclusive dose range of 10 µg/mL to 750 µg/mL, cell survival ranged from 103% to 1% in a somewhat erratic fashion relative to the dose. However, no dose-related or repeatable increases in mutant frequency were detected.

CHO cells were examined also for cytogenetic damage in assays for CPFB-induced chromosomal aberrations and sister chromatid exchanges (SCEs). Excessive toxicity occurred at 500 µg/mL with or without S9, and cell cycle delay (indicating toxicity) was observed at 200 µg/mL without S9. Accordingly, a dose range of 50 µg/mL to 200 µg/mL was analyzed without S9, using a normal harvest

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(10 h) and a delayed harvest (20 h) at 200 µg/mL. No increases in chromosomal aberrations were observed. With two-hour treatments with S9, 300 µg/mL of CPFB was toxic, and a dose range of 100 to 250 µg/mL was analyzed. Again, no increases in aberrations were found. The assay for SCE showed no dose-related effects for doses up to 167 µg/mL in absence of S9 but an apparent response with S9, beginning at 16.7 µg/mL. A repeat trial with S9 demonstrated a dose-related increase in SCE of 24% to 82% over the solvent control for the dose range of 100 to 250 µg/mL. Therefore, under S9 activation conditions CPFB was active in causing genetic changes in CHO cells as indicated by SCE.

CPFB was assayed for its ability to induce morphological transformation *in vitro* in cultures of mouse BALB/c-3T3 cells, both in the presence and absence of a rat liver S9 metabolic activation system. The treatment periods were two hours with S9 and 72 h without S9. The two test conditions resulted in similar toxicities, as measured by the clonal survivals of ouabain-resistant cells in the presence of a wildtype monolayer cultures. A survival range of 112% to 0% was obtained for a CPFB concentration range of 50 to 600 µg/mL. The number of transformed foci was not increased by any of the treatments, so CPFB had no detectable activity as a transforming agent to BALB/c-3T3 cells.

An *in vivo* assessment of genetic activity was performed by dosing male F-344 rats with four doses of CPFB ranging from 625 mg/kg to the maximum amount of 5000 mg/kg. Four to five hours after single oral administrations, primary hepatocyte cultures were established to determine the degree of DNA repair (unscheduled DNA synthesis) by labeling with ^{3}H-thymidine. An autoradiographic analysis of the extent of nuclear labeling and percent of cells in DNA repair showed no increases over cultures from control rats. Therefore, no evidence was obtained for DNA damage in the liver caused by the CPFB treatments.

The results of the above genetic tests indicate that CPFB has little or no ability to interact with genetic material. For five of the six genetic endpoints, no responses were obtained. The response obtained for SCEs only in the presence of S9 activation was somewhat at variance with the other tests, which indicated little or no effect on CPFB by the S9 metabolic system. The SCE assay is known to be a very sensitive in vitro test for chemical interaction with chromatin structure (by direct or indirect actions) and is of relatively minor significance in the absence of detectable activity at other endpoints. Damage to DNA in rat liver was not detectable by the direct assay for unscheduled DNA synthesis, even following the excessive dose of 5000 mg/kg. Thus, the results of the genetic test battery would predict little or no genetic risk from CPFB.

SECTION 8

APPENDIX A

SUMMARY OF TESTING ON CHLOROPENTAFLUOROBENZENE WITH RAT PRIMARY HEPATOCYTES

Cifone, M.A.[a]

ABSTRACT

An attempt was made to evaluate chloropentafluorobenzene (CPFB) in the *In Vitro* Rat Primary Hepatocyte Unscheduled DNA Synthesis (UDS) Assay. The test material required that testing be performed in closed containers. Several containers and conditions were tried and none resulted in acceptable levels of survival. While conditions for the UDS assay were being developed, an attempt was made to obtain toxicity information. Three cytotoxicity assays were initiated but the data from the first studies were unacceptable. The second study demonstrated that the test material was excessively toxic to rat primary hepatocytes at concentrations at and above 500 µg/mL. In the third study, moderate toxicity was observed at 50.1 µg/mL and lower concentrations were nontoxic. An attempt was made to perform the UDS assay. Hepatocytes from two rats were exposed to CPFB at concentrations from 2.50 to 501 µg/mL. Erratic toxicity curves were observed and the cells had morphologies that were unacceptable for analysis. The studies were terminated because acceptable conditions for performing the UDS assay would require further developmental studies.

OBJECTIVE

The objective of this assay was to detect DNA damage caused by the test material, or an active metabolite, by measuring UDS in rat primary hepatocytes *in vitro*. The existence and degree of DNA damage was inferred from an increase in net nuclear grain counts in treated hepatocytes when compared to untreated hepatocytes. The types of DNA damage are unspecified but must be recognizable by the cellular repair system and result in the incorporation of new bases (including $^{3}H2$-thymidine) into DNA

RATIONALE

Fresh hepatocytes obtained from rat liver will attach to a surface in culture and continue to metabolize for several days without undergoing cell division. Only a small percentage of the cells enter S-phase (replicative DNA synthesis). Therefore, if ^{3}H-thymidine is introduced in the culture medium, little or no label will be incorporated into nuclear DNA. The addition of a test material that interacts with the DNA often stimulates a repair response in which the altered portion of DNA is

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excised and the missing region replaced by DNA synthesis. This synthesis of DNA by nondividing cells is known as UDS and can be measured by determining the amount of ^{3}H-thymidine incorporation using an autoradiographic method. Cells involved in DNA replication are recognized by heavy labeling of the nuclei and are excluded from the evaluation of UDS activity. Autoradiographic measurement of DNA repair is highly sensitive and appears to correlate very well with the known mutagenic or carcinogenic activities of chemicals (Williams, 1977). Furthermore, the use of primary hepatocytes has the advantage that these cells have sufficient metabolic activity to eliminate the need for the addition of a microsomal activation system.

MATERIALS

Indicator Cells

The indicator cells for these assays were hepatocytes obtained from adult male F-344 rats (150-300g), purchased from Charles River Breeding Laboratories, Incorporated. The animals scheduled for this assay were fed Purina Certified Rodent Chow (Formula 5002) and water ad libitum. Two animals, identified by cage card, were used for each trial of the UDS assay after a minimum quarantine period of five days. One rat was used for each cytotoxicity assay.

The cells were obtained by perfusion of the liver *in situ* with a collagenase solution (see Section entitled "UDS and Cytotoxicity Assays"). Monolayer cultures were established on plastic coverslips in culture dishes and were used the same day for initiation of the UDS assay. All cultures were maintained as monolayers at about 37°C in a humidified atmosphere containing approximately 5% CO_2.

Medium

The cell cultures were established in Williams' Medium E supplemented with 10% fetal bovine serum, 2mM L-glutamine, 100 µg/mL streptomycin sulfate, and 150 µg/mL gentamicin (WME+). In some cases 25 mM Hepes buffer (pH 7.2) was included in the medium. After the establishment period, the serum was removed. This latter culture medium is referred to simply as WME1. This also contained Hepes buffer. If Hepes buffer was not added, the cultures were blanketed with 5% CO_2 to maintain the correct pH range

Controls

Negative control

A negative control consisting of assay procedures performed on cells exposed only to the test material solvent was performed. The solvent for the UDS assay was deionized water containing 1.0% F-68 Pluronic®. The final concentration of solvent in the medium was 10%.

An untreated control consisting of assay procedures performed on mock-exposed cells was also included.

Positive Control

The positive control compound is known to induce UDS in rat hepatocyte primary cell cultures. 2-Acetylaminofluorene (2-AAF) at 4.48×10^{-7}M (0.10 µg/mL) was used as the positive control.

EXPERIMENTAL DESIGN

Dosing Procedure

The test material was dissolved at the highest desired concentration in deionized water containing 10% F-68 Pluronic®. CPFB was immiscible with the solvent and was dispersed with a Tissumizer® to obtain a milky white suspension. Lower concentrations were then prepared by dilution with 10% F-68® (initial studies) or 1% F-68® (later studies including UDS assay). The dosing treatments were prepared by performing 1:10 dilutions of the stocks into WMEI containing ^{3}H-Tdr (final concentration, 5 µCi/mL).

Fresh preparations of test material in the vehicle were used for the biological testing. Treatments were initiated by replacing the medium on the cell cultures with WMEI containing the test material at the desired concentrations and 5 µCi/mL ^{3}H-thymidine (20 Ci/mmole).

Dose Selection

Preliminary cytotoxicity assays were performed to determine the doses to be initiated in the UDS assay. The initial cytotoxicity assay was initiated with treatments from about 5000 to about 50.0 µg/mL. The concentrations tested were lowered when it was observed that the test material was highly toxic. Three cytotoxicity assays were performed.

UDS and Cytotoxicity Assays

This assay was based on the procedures described by Williams (1977, 1980). The hepatocytes were obtained by perfusion of livers *in situ* for about four minutes with Hanks' balanced salts (Ca^{++} - Mg^{++}-free) containing 0 5 mM ethyleneglycol-bis (-aminoethyl ether)-N, N-tetraacetic acid (EGTA), and HEPES buffer at pH 7.2. Then WMEI containing 50-100 units/mL of collagenase was perfused through the liver for about ten minutes. The hepatocytes were obtained by mechanical dispersion of excised liver tissue in a culture dish containing the WMEI culture medium and collagenase. Clumps of cellular tissue and debris were removed by allowing the clumps to settle to the bottom of the plate. The supernatant was centrifuged and the cell pellet resuspended in WME +.

After obtaining a viable cell count, a series of 8 dram Shell vials (some containing a 15-mm round, plastic coverslip) was inoculated with viable cells in WME +.

An attachment period of 1.5 to 2 h at approximately 37°C in a humidified atmosphere containing about 5% CO_2 was used to establish the cell cultures. Unattached cells were then removed and the cultures were refed with WMEI. The assays were initiated within three hours by replacing the media in the vials with WMEI containing 5 µCi/mL ^{3}H-thymidine, (20 Ci/mmole) and the test material at the desired concentration. Cytotoxicity assays were initiated without the added label. After treatment for 18 to 20 h, the assays were terminated by washing the cell monolayers twice with WMEI. For the cytotoxicity assays, two cultures were used to monitor toxicity. For the UDS assay, three of the cultures from each treatment were washed with WMEI containing one mM thymidine and were further processed as described below. Another two cultures used to monitor the toxicity of each treatment were refed with WMEI and returned to the incubator. At 20 to 24 h after the initiation of the treatments, viable cell counts (trypan blue exclusion) were determined to estimate cell survival relative to the negative control.

The nuclei in the labeled cells were swollen by addition of 1% sodium citrate to the coverslips for eight to ten minutes, and then the cells were fixed in acetic acid:ethanol (1:3) and dried for at least twenty-four hours. The coverslips were mounted on glass slides, dipped in an emulsion of Kodak NTB2, and dried. The coated slides were stored for seven to ten days at 4°C in light-tight boxes containing packets of Drierite. The emulsions were then developed in D19, fixed, and stained using Williams' modified hematoxylin and eosin procedure.

The cells were examined microscopically at approximately 1500 x magnification under oil immersion and the field was displayed on the video screen of an automatic counter. UDS could not be measured because of the morphological appearance of the cells.

RESULTS

The test material, CPFB, was insoluble in medium and formed large globules that settled to the bottom of the vessel. In order to disperse the test material in the solvent and allow even exposure of the target hepatocyte cells, 10% F-68 Pluronic® (w/v in water) was used to suspend the test material. CPFB was mixed with 10% F-68 Pluronic® and the test material was dispersed using a Tissumizer®. A milky white suspension formed that appeared to be stable. The highest concentration prepared was 50 mg/mL and, in most cases, this suspension was diluted with 10% F-68 Pluronic® to obtain a series of stock solutions. In some of the later experiments, the 10% F-68 Pluronic® was reduced to 1% (for diluting the stocks) in order to reduce any possible effects of the Pluronic® on the hepatocytes. The final dosing concentrations were prepared by performing 1:10 dilutions of the F-68 Pluronic® stocks with WME medium containing ^{3}H-Tdr (final concentration, 5 µCi/mL). No labeled thymidine was

added to the treatments used for cytotoxicity tests. The test material formed a good suspension in the medium.

The cytotoxicity and UDS assays were initiated by replacing the medium on the cells with medium containing the appropriate concentration of test material (and ^{3}H-Tdr, if appropriate).

Because of the volatile nature of CPFB, and in order to maintain a stable and representative concentration of the test material during the dosing period, it was necessary to perform the assays in closed containers. The hepatocytes were seeded into 8 dram glass Shell vials containing 15 mm polyester coverslips instead of the usual 35 mm plastic culture dishes containing 25 mm round polyester coverslips. In the early experiments, 25 mm Hepes buffer was included in the medium to maintain the appropriate pH. It was later found to be slightly toxic and the cultures were blanketed with 5% CO_2, which also maintained the appropriate pH. The use of closed containers proved to be a continual problem throughout the studies. A brief description of studies performed to alleviate this problem is shown in Appendix B.

To select the appropriate doses for the UDS assay, it was decided that a preliminary cytotoxicity assay would be performed. This is not usually necessary for this assay but was instituted because of the special conditions and the request for a two-rat study. Trial 1 was initiated with treatments from about 5000 to about 50.0 µg/mL. This assay was terminated because all the cells were dead, even the solvent control cells. The controls in Trial 2 were better but all doses (50,000 to 500 µg/mL) were dead. Trial 3 (Table 8-A-1) was initiated at lower concentrations (1000 to 1.00 µg/mL). The test material was moderately toxic at 50.1 µg/mL and lower concentrations were nontoxic.

The UDS assay was initiated with cells from two rats at concentrations from 500 to 2.5 µg/mL. The cytotoxicity curve was erratic in both studies and the cells were unacceptable for analysis. The assay was terminated.

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TABLE 8-A-1. SUMMARY OF DATA FROM TRIAL 5 OF THE RAT HEPATOCYTE CYTOTOXICITY ASSAY USING CHLOROPENTAFLUOROBENZENE

Test Condition	Concentration	Survival at 20.1 h (%)***
Negative Control media	------	43.1
Solvent Control 1% F-68	10%	100.0
Test Material:		
	1000 µg/mL	0
	501 µg/mL	0
	100 µg/mL	0
	50.1 µg/mL	69.7
	10.0 µg/mL	100.0
	1.00 µg/mL	162.4

***Survival = Number of viable cells per unit area relative to the solvent control

APPENDIX B

SUMMARY OF ATTEMPTS TO PERFORM THE UDS ASSAY IN CLOSED CONTAINERS

Three UDS studies were requested that required the assays be performed in closed glass containers. This is difficult in the UDS assay because hepatocytes do not attach well to glass and do not survive well in closed containers. The requirement for glass coverslips was waived when the polyester coverslips routinely used were shown to be resistant to the chemicals. However, it was still a requirement that closed containers be used. A preliminary experiment was performed to determine the technical problems associated with this requirement.

In the preliminary experiment, several glass containers containing coverslips were seeded with cells; Hepes buffer was added to some of the cultures to control the pH. The cells attached very well after 1.5 to 2 h and the toxicity tests were initiated using Shell vials. When the toxicity tests were performed, no cells were alive 18 to 20 h later. It appeared that cell attachment was normal but long term survival was a problem. Several experiments were then performed to determine the parameters that affected survival. The following changes were made:

- Hepes buffer was not used and the cultures were gassed with 5% CO_2 to maintain an acceptable pH.
- The culture vessels were pretreated with medium containing 20% serum.
- The number of cells seeded was reduced.
- The volume of medium used was increased.
- Other culture vessels were tried again but rejected because they were not currently available or because of technical reasons.
- The dosing and feeding procedures were changed to make the cell handling more gentle.

Cytotoxicity studies were initiated again but were only partially successful. Additional studies were performed and the following changes were made:

- 1% serum was added to the dosing medium.
- At least twice the number of cultures was set up.
- The requirement for 50% survival of the cells in the control cultures was waived.

The cytotoxicity tests were again initiated and were partially successful and doses could be chosen. The UDS assays were initiated.

In the UDS assays initiated, the results were variable. In some cases, cells appeared to survive but the toxicities were erratic. Slides were prepared and autoradiography was performed. When the slides were developed, UDS could not be determined because most of the cells were rounded and cell labeling was abnormal. In addition, all of the positive control cultures were excessively toxic. None of the changes that were instituted avoided the problem of random loss of cultures or whole dose groups. It appeared that the cells were more fragile under conditions where closed containers were used and the parameters involved were not readily controlled. Reliable results could not be obtained from the cells under the conditions requested

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